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Multiplexed mGRASPi for scalable mapping of synaptic convergence

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Abstract

Convergence of distinct synaptic inputs underlies the integration of diverse information within individual neurons, yet how multiple input populations converge remains difficult to map. Mesoscale tracing lacks synaptic resolution, while existing synapse-labeling methods are limited to one or two pathways. Here we present multiplexed mGRASPi, which detects synaptic contacts through transsynaptic reconstitution of complementary split-GFP fragments and assigns their presynaptic origins by combinatorial epitope encoding, together with SynScope, an automated framework for decoding these labels in densely intermingled circuits. Using conventional four-channel fluorescence imaging, we distinguished synapses from up to 13 input populations converging onto individual neurons in hippocampal and cortical circuits. In the dentate gyrus, multiplexed mGRASPi with SynScope resolved structured variation in the composition of convergent inputs across layers and individual cells, beyond the classical laminar organization of afferent projections. This integrated framework enables scalable, single-synapse analysis of how diverse inputs converge onto individual neurons.

Introduction

Convergence of distinct synaptic inputs is a central organizing principle of neural circuits. Individual neurons integrate signals from multiple presynaptic populations, and the identity, number and spatial distribution of their synapses define the structural substrate for dendritic integration and postsynaptic output[1-3]. Defining how distinct inputs converge onto single neurons is therefore essential for understanding circuit computation. In recent years, large-scale circuit mapping has advanced rapidly through mesoscale projection mapping[4-7], viral circuit tracing[8-12], single-cell reconstruction[13-18], and emerging barcode-based connectivity approaches[19-21]. These approaches have revealed projection-defined input organization across brain regions, but do not delineate convergence at the level of defined synaptic contacts on single postsynaptic cells.

At synaptic resolution, convergence mapping relies primarily on electron microscopy (EM)[22-26] and genetically targeted fluorescence reconstitution strategies[27-32]. EM provides nanometer-scale ultrastructure and increasingly large connectivity volumes, but assigning multiple predefined molecular or projection identities to presynaptic partners remains challenging at scale. Fluorescence reconstitution approaches such as mammalian GFP reconstitution across synaptic partners (mGRASP)[28] and related variants bridge molecular specificity with optical accessibility by visualizing genetically defined presynaptic–postsynaptic contacts through split fluorescent protein (split-FP) complementation. Most existing split-FP systems are designed to interrogate one defined presynaptic–postsynaptic pairing at a time. Multiplexing has generally relied on spectrally distinct split-FP pairs, coupling each additional input identity to an additional compatible fluorophore[29,30,32]. Although the repertoire of split-FPs continues to expand[33], only a limited subset exhibits efficient complementation and sufficient signal for synaptic labeling. Consequently, existing fluorescence reconstitution strategies can visualize defined synapses but remain limited in resolving many input populations simultaneously on individual postsynaptic neurons.

Rather than expanding the number of synaptic split-FP pairs, we restructured the logic of synaptic encoding. We developed multiplexed mGRASPi (mGRASP improved), which combines an improved presynaptic carrier for efficient axonal distribution with a multiplex-compatible architecture in which synapse detection is decoupled from input identity. Synaptic contacts are detected exclusively through transsynaptic complementation of a single high-efficiency split-sfGFP pair[33,34], whereas presynaptic populations are independently encoded by combinations of epitope tags and decoded using conventional multichannel fluorescence imaging. This separation allows the number of resolvable input identities to expand combinatorially without requiring additional split-FP pairs. To analyze and inspect the resulting volumetric datasets, we developed SynScope, an integrated, interactive computational framework that corrects imaging variation, segments mGRASPi puncta and classifies their presynaptic identities from epitope combinations. Using this framework, we mapped synapses from up to 13 defined input

populations in hippocampal and cortical circuits and resolved structured convergence patterns across layers and among individual dentate granule cells. These applications establish a scalable route from projection-level mapping to single-synapse analysis of input convergence onto individual neurons, providing a structural basis for investigating the functional organization of neural circuits.

Results

Design of multiplexed synaptic convergence mapping

Conventional split-FP strategies couple synapse detection to input identity, requiring a spectrally distinct, efficiently complementing split-FP pair for each input population (**Fig. 1a**). To overcome this constraint and enable scalable convergence mapping, we decoupled synapse detection from presynaptic identity and implemented a combinatorial epitope-encoding strategy (**Fig. 1a–c**). Synaptic contacts were detected by transsynaptic reconstitution of a common split-sfGFP pair, whereas presynaptic populations were independently identified by binary combinations of epitope tags. Four binary epitope channels, compatible with conventional confocal microscopy, theoretically generate 15 (2^4–1) non-zero combinatorial codes. Because synaptic, postsynaptic and epitope signals were acquired together within the same image stack, we excluded the green-only and red-only codes to avoid ambiguity with reconstituted split-sfGFP and postsynaptic signals, respectively. This design therefore enables simultaneous identification of up to 13 distinct presynaptic populations within a single preparation without requiring spectrally distinct split-FP pairs or spectral unmixing (**Fig. 1b**).

The original mGRASP components were engineered as chimeric membrane proteins to localize split-GFP fragments at synapses while minimizing nonspecific reconstitution and perturbation of endogenous synaptic organization. Although this architecture has supported synapse mapping across diverse local and long-range circuits[28,30,32,35-41], multiplexing many input populations within a single preparation requires consistent presynaptic delivery across inputs differing in projection range and neurotransmitter identity. We therefore retained the original chimeric design principle but redesigned the presynaptic carrier to improve trafficking to distal terminals (**Fig. 1c and Methods**). We engineered a VAMP2-based pre-mGRASPi scaffold, leveraging its intrinsic synaptic-vesicle trafficking to promote axonal transport and presynaptic enrichment[42,43]. To minimize perturbation of endogenous presynaptic function, we truncated the VAMP2 coiled-coil domain (Δ61–80) and introduced the M46A mutation to reduce endocytic retrieval[44]. A PDZ-binding motif derived from the C terminus of neurexin-1β (Nx+10) was added to promote synaptic localization[45]. Split-GFP11 was positioned after the VAMP2 transmembrane domain, allowing its exposure to the synaptic cleft upon vesicle exocytosis and complementation with postsynaptic GFP1–10.

For presynaptic identity encoding, tandem copies of V5, HA, Ollas and Myc epitopes were incorporated into modular pre-mGRASPi variants, either individually or in defined combinations. Tag placement and copy number were selected to preserve membrane trafficking and antibody accessibility (Fig. 1c). The blue fluorescent reporter used in the original pre-mGRASP was removed to reserve the blue channel for combinatorial epitope detection. We also maintained a modular design, enabling incorporation of additional functional effectors through self-cleaving 2A-linked expression for targeted circuit manipulation. The resulting construct collection, summarized in **Table 1**, provides adaptable configurations for circuit-specific labeling and manipulation. Finally, we incorporated a destabilization domain derived from the well-characterized d1-GFP (~1 h half-life)[46], designed to limit synaptic accumulation and persistence of reconstituted signals and thereby improve temporal resolution.

Improved presynaptic targeting and synapse-level validation across transmitter-defined circuits

We first evaluated axonal targeting of the redesigned pre-mGRASPi by comparison with spaghetti monster fluorescent protein (smFP), a reporter that efficiently labels fine neuronal structures[47]. Pre-mGRASPi showed efficient expression of distal projections, including corticothalamic axons and contralateral Schaffer collaterals, with signal intensities up to 6.5-fold higher than those obtained with smFP in long-range pathways (**Extended Data Fig. 1**). Differences were modest in short-range projections but increased with projection distance, and distal pre-mGRASPi-labeled axons contained bouton-like puncta consistent with presynaptic enrichment. We next assessed synaptic labeling in the hippocampal CA3–CA1 pathway. AAV-mediated expression of pre-mGRASPi in CA3 pyramidal neurons and post-mGRASP in CA1 pyramidal neurons produced discrete reconstituted GFP puncta along CA1 dendrites at sites of axon–dendrite apposition (**Fig. 1d–f**). High-resolution three-dimensional Airyscan imaging resolved reconstituted signals at contacts between presynaptic boutons and dendritic spines. Protein-retention expansion microscopy further showed that mGRASPi signals were positioned between juxtaposed Bassoon and Homer1 signals[48] (**Fig. 1f**). Together, these observations validate the synapse-level specificity of mGRASPi reconstitution.

Compatibility across transmitter-defined pathways was examined in glutamatergic cortical, GABAergic pallidal, dopaminergic nigral and serotonergic raphe projections. Reconstituted puncta were detected along axons labeled by the corresponding transmitter markers (**Extended Data Fig. 2**). More broadly, mGRASPi labeled synaptic contacts across 27 distinct circuits spanning hippocampal, cortical and basal ganglia networks (**Extended Data Fig. 3**), supporting its use across projection classes and transmitter systems.

Multiplexed resolution of presynaptic populations

Having established the combinatorial encoding strategy, we next examined whether multiplexed mGRASPi could resolve multi-input synaptic convergence within a single preparation. We generated a panel of pre-mGRASPi variants carrying single-, double-, triple- and quadruple-epitope combinations of V5, HA, Ollas, and Myc (**Table 1**; constructs available from Addgene). Because detection performance depended on both epitope identity and the fluorophore used for secondary-antibody detection, we systematically quantified signal-to-noise ratios and depth-dependent signal attenuation for each epitope across the four imaging channels (**Extended Data Fig. 4**). These measurements guided channel assignment in subsequent experiments: less readily detected epitopes were paired with their best-performing channels, whereas robust epitopes could be assigned flexibly.

We next tested the complete code set *in vivo* using dentate granule cell mossy fiber projections to the stratum lucidum of CA3, where large presynaptic boutons provided a tractable setting for direct visualization of combinatorial epitope signals (**Fig. 1g**). We delivered 13 AAV vectors, each encoding a distinct epitope combination, to spatially separated regions of the granule cell layer (GCL), thereby generating 13 spatially defined presynaptic populations within a common projection pathway. All 13 presynaptic identities were distinguished simultaneously in the same tissue (**Fig. 1h,i**). Overview imaging showed the topographic organization of mossy fiber projections across CA3, while higher-magnification imaging distinguished individual boutons by their combinatorial epitope signatures. These results validate the full 13-code capacity of multiplexed mGRASPi in intact brain tissue.

Synaptic convergence mapping in multi-input circuits with multiplexed mGRASPi

We next applied multiplexed mGRASPi to map synaptic convergence in defined multi-input circuits. We first targeted dentate GCs, which receive multiple anatomically and functionally distinct inputs with classically described laminar preferences (**Fig. 2a–c**). We labeled 4 well-characterized inputs to DG GCs—the lateral entorhinal cortex (LEC), medial entorhinal cortex (MEC), supramammillary nucleus (SuM), and contralateral hilus containing mossy cells (MCs)—using pre-mGRASPi variants carrying distinct combinatorial epitope codes. Each presynaptic input showed characteristic laminar enrichment, and reconstituted GFP puncta marked synaptic contacts between epitope-defined axons and postsynaptic GCs (**Fig. 2b,c**). This four-input DG dataset formed the basis for the SynScope decoding pipeline described below and for subsequent layer- and cell-level analyses of synaptic convergence.

We then challenged the approach in the medial prefrontal cortex (mPFC), where anatomically and neurochemically diverse long-range inputs intermingle within the same cortical domain. Post-mGRASP was expressed in mPFC neurons, while pre-mGRASPi variants carrying 13

distinct epitope codes labeled 13 presynaptic populations (**Fig. 2d,e**). These comprised 9 glutamatergic cortical, limbic and thalamic inputs: the contralateral infralimbic (contra. IL), prelimbic (contra. PL) and anterior cingulate (contra. ACC) cortices; the dorsal and ventral divisions of the basolateral amygdala (BLAd and BLAv); ventral CA1 (vCA1); agranular insula (AI); and the mediodorsal (MD) and ventromedial (VM) thalamic nuclei. The remaining 4 were modulatory inputs comprising dopaminergic projections from the medial and lateral divisions of the ventral tegmental area (VTAm and VTAl), serotonergic projections from the dorsal raphe (DR) and cholinergic projections from the basal forebrain (BF). Implementing 13-input convergence mapping in the densely innervated mPFC required careful configuration of epitope–channel combinations. We configured the code assignments by integrating the epitope calibration described above with the projection range and axonal density of each input. Because dense cortical innervation also made reconstituted GFP puncta difficult to distinguish from epitope-labeled axonal boutons, we spectrally separated the synaptic and axonal signals using 488-nm and 514-nm excitation, respectively. With this configuration, synaptic contacts from all 13 anatomically and neurochemically distinct input populations were simultaneously mapped within the same cortical domain. Higher-resolution imaging further distinguished contacts made by multiple presynaptic populations along individual dendritic branches, thereby resolving their convergence onto individual mPFC neurons (**Fig. 2f**). We consolidated these calibration and implementation considerations into practical guidelines for selecting epitope–channel configurations according to the number of inputs, projection range and axonal density (**Extended Data Fig. 5**). Together, these applications demonstrate that multiplexed mGRASPi maps the convergence of multiple defined inputs onto individual neurons at single-synapse resolution across both laminar and densely intermingled circuit architectures.

SynScope for decoding multi-input synaptic convergence

Multiplexed mGRASPi produces multichannel volumetric images in which reconstituted synaptic puncta, postsynaptic dendrites and epitope-labeled presynaptic boutons are densely intermingled. Because each presynaptic identity is determined by a binary epitope pattern, depth-dependent signal attenuation, field-dependent intensity variation and even submicron chromatic displacement between channels can generate incorrect codes. We therefore developed SynScope, an integrated computational framework for image preprocessing, synapse detection and presynaptic identity assignment (**Fig. 3a**).

Reliable binary decoding required bouton-scale spatial alignment and stable signal intensity throughout the imaging volume. SynScope therefore corrects field-dependent intensity variation introduced by tiled acquisition, performs submicron chromatic registration between channels and normalizes z-dependent signal loss arising from differential antibody penetration and wavelength-dependent attenuation using an adaptation of our previously described Interdependent Self-Cooperative Learning (ISCL) method[49] (**Fig. 3b–d, Extended Data Fig. 6, and Methods**). SynScope

next decodes presynaptic identity within synapse-centered regions of interest (ROIs), ensuring that epitope signals are evaluated specifically at sites of mGRASPi reconstitution. mGRASPi-labeled synapses are first detected using an established mGRASP-based algorithm[50,51], and an ROI is defined around each synapse. Within each ROI, morphology-based filtering removes continuous dendritic signals, and Gaussian mixture model–based adaptive thresholding converts epitope signals into binary channel patterns (**Fig. 3e and Extended Data Fig. 7a**).

Binary channel patterns alone cannot distinguish true co-expression of multiple epitopes within a bouton from apparent co-localization produced when independently labeled axons cross within the optical resolution limit. SynScope therefore quantifies the fractional spatial overlap between each pair of epitope signals within a synapse-centered ROI and evaluates whether those signals exhibit consistent bouton morphology and axonal continuity. These measurements form the basis of a hybrid decoding strategy combining biologically informed rules with supervised machine learning. Graph-based association first links signal pairs satisfying the spatial and morphological criteria to infer candidate epitope codes. A supervised machine-learning classifier (LightGBM) then integrates the resulting quantitative and morphology-derived features to assign the presynaptic identity of each synapse[52] (**Fig. 3f-h**).

Using expert annotations as the reference, SynScope achieved 91.4% accuracy in the 4-input DG dataset and 91.6% in the 13-input mPFC dataset, maintaining high performance across increasing combinatorial complexity (**Fig. 3i**). By contrast, machine-learning models using only channel intensities achieved less than 50% accuracy, underscoring the importance of SynScope's synapse-centered, biologically informed features. Ablating field-dependent intensity correction or chromatic registration lowered accuracy to 62.3% and 67.7%, respectively, whereas removing z-dependent normalization reduced it to 33.7%, the largest decline among the preprocessing steps. Replacing adaptive local thresholding with global thresholding yielded 32.2% accuracy (**Fig. 3i**). Together, these results demonstrate that robust combinatorial decoding requires coordinated volumetric signal correction, synapse-centered adaptive thresholding and biologically informed classification. To facilitate broader use, SynScope is provided through a web-based platform (http://synscope-mgrasp.com, access code: synscope) that integrates preprocessing, decoding and interactive inspection of assigned synapses, with server-side execution enabling reproducible analysis without local software installation (**Extended Data Fig. 8 and Table 2**). The complete workflow is illustrated in **Supplementary Movie 1**.

Synapse-resolved convergence in the mPFC

Having established the accuracy of SynScope decoding, we used its assignments to examine multi-input convergence at cellular and laminar scales. Using the 13-input mPFC preparation shown in **Fig. 2d,e**, SynScope mapped mGRASPi-labeled synapses from multiple

presynaptic populations across the dendritic arbor of a sparsely labeled layer 5 neuron. Three-dimensional reconstruction displayed the decoded input identities directly on the postsynaptic morphology (**Fig. 4a**). At higher magnification, individual synapses along the same dendritic branch were assigned to different presynaptic populations despite the densely intermingled surrounding projections (**Fig. 4b**). This reconstruction provided a subcellular view of multi-input convergence that could not be inferred from axonal projection patterns alone. At the circuit scale, we quantified input-specific synapses across cortical layers. Synapses from all 13 presynaptic populations were detected across the mPFC rather than being confined to discrete laminae (**Fig. 4c**). Although several inputs showed modest laminar preferences, no input population was restricted to a single layer, and each layer contained synapses from multiple cortical, subcortical and neuromodulatory sources. Together, these analyses demonstrate that SynScope maps multi-input synaptic convergence across cortical layers, individual neurons and dendritic branches within a densely intermingled circuit.

Laminar and compositional organization of multi-input convergence onto DG GCs

Having demonstrated synapse-resolved convergence mapping in the densely intermingled mPFC, we next focused on DG GCs, a defined postsynaptic population receiving functionally distinct afferents, to compare multi-input convergence across dendritic layers and among individual cells. Using multiplexed mGRASPi and SynScope, we mapped input-specific synapses along the dendritic arbors of 125 individually reconstructed GCs (**Fig. 5a**). Synapse density for each of the 4 mapped inputs varied substantially among cells (**Fig. 5b**). Pairwise comparisons showed that MEC density correlated with LEC and MCs densities ($r = 0.72$ and 0.66, respectively), whereas LEC and SuM densities showed no correlation ($r = -0.05$; **Fig. 5c**). These analyses revealed substantial cell-to-cell variation in synapse density, with some input pairs covarying across GCs and others showing little or no correlation. We next examined how the classical laminar organization of DG afferents was represented at the level of identified synapses. While MCs, MEC and LEC synapses retained their expected enrichment in the inner molecular layer (IML), middle molecular layer (MML) and outer molecular layer (OML), respectively, each extended beyond its predominant layer (**Fig. 5d–g**). Layer-specific analysis confirmed that the IML and OML were dominated by MCs (87.1%) and LEC (81.9%) inputs, respectively. In contrast, the MML, classically associated with MEC input, contained a mixed composition of MEC (50.3%), LEC (25.6%) and MCs (24.1%) synapses and exhibited the highest overall synapse density (**Fig. 5e**).

Because individual GCs integrate information carried by distinct afferents, their relative synaptic contributions represent an important feature of convergence. We therefore quantified the contribution of each input as a fraction of total mapped synapse density. Ordering GCs by MCs fraction revealed marked cell-to-cell differences in input composition (**Fig. 5h**). MCs and LEC

fractions were strongly anticorrelated (r = –0.89), whereas MCs and SuM fractions were positively correlated (r = 0.32) and LEC and SuM fractions negatively correlated (r = –0.50); MEC showed comparatively weak correlations with the other inputs (**Fig. 5i**). The corresponding four-input profiles showed that MEC contributions remained comparatively consistent across the population (**Fig. 5j**). Principal component analysis (PCA) of centered log-ratio (clr)-transformed compositions identified a dominant axis characterized by opposing LEC (–0.75) and SuM (+0.63) loadings, with a smaller contribution from MCs (+0.19) and little contribution from MEC (–0.06; **Fig. 5k**). Together with the pairwise correlations, this pattern indicates that MEC forms a comparatively stable component of the four-input profile, whereas the dominant cell-to-cell variation reflects opposing relative contributions of LEC and MCs/SuM inputs. Because input fractions sum to one, opposing relationships can arise partly from this mathematical constraint. We therefore compared observed log-ratio distributions with null distributions generated under a shared input composition to assess whether the variation exceeded that expected from counting noise (**Fig. 5l**). The observed spreads of log(LEC/SuM) and log(LEC/ MCs) were 1.61 and 1.28, respectively, compared with null spreads of approximately 0.46 and 0.27. These differences indicate that cell-to-cell variation exceeded that expected from counting noise alone. Together, by resolving synaptic intermixing across dendritic layers and cell-level input composition, multiplexed mGRASPi and SynScope extend classical projection-defined maps to a single-synapse view of how multiple defined afferents converge onto individual neurons.

Discussion

Multiplexed mGRASPi and SynScope provide an integrated molecular and computational framework for mapping the convergence of multiple defined afferents onto individual neurons at single-synapse resolution. By separating split-sfGFP-based synapse detection from combinatorial encoding of presynaptic identity, the framework resolved up to 13 input populations within a single tissue preparation using conventional multichannel fluorescence imaging. Applications across hippocampal and cortical circuits demonstrated multiplexed labeling across projection ranges, neurotransmitter systems and densely intermingled axons. In the DG, the framework showed that classical laminar preferences coexist with synaptic intermixing within dendritic layers. Across individual GCs, the MEC fraction remained comparatively stable, whereas the LEC contribution varied inversely with those of MCs and SuM. The advance therefore lies not simply in greater multiplexing, but in extending circuit mapping from projection-defined territories to the synaptic composition of inputs converging onto individual neurons.

Spectrally multiplexed GRASP variants typically distinguish two presynaptic populations and have been extended to three using spectral unmixing, but further scaling remains constrained by the limited repertoire of spectrally separable and efficiently complementing split-FPs[29,30,32].

Multiplexed mGRASPi instead shifts input identity from the spectral domain to combinatorial epitope encoding, allowing a common split-sfGFP pair to detect synapses while epitope combinations identify their presynaptic origins. The newly engineered presynaptic scaffold further supports reliable implementation across inputs differing in projection range and neurotransmitter identity. Accurate multiplexed decoding requires bouton-scale fidelity, as even submicron spatial misalignment or depth-dependent intensity variation can generate incorrect binary codes. Accordingly, SynScope combines integrated volumetric preprocessing with synapse-centered decoding based on biologically informed rules and supervised machine learning. Although developed for mGRASPi, these computational principles address general challenges in volumetric immunofluorescence imaging and may be adaptable to other multiplexed antibody-based datasets.

Synapse detection across 27 diverse circuits, together with the simultaneous mapping of 13 anatomically and neurochemically distinct inputs in the mPFC, demonstrates the breadth of the framework. Across the densely intermingled mPFC and the classically laminated DG, synapse-resolved mapping revealed patterns of afferent convergence onto individual neurons that were not captured by projection organization alone. Whereas projection maps describe the regional and laminar distributions of labeled axons, synapse-resolved convergence maps reveal the composition and spatial organization of defined afferent inputs at the level of individual neurons, providing a detailed structural substrate for investigating how multiple streams of information are integrated. In the DG, a comparatively stable MEC contribution, together with inverse covariation between LEC and MCs/SuM inputs, revealed structured cell-to-cell variation in convergent input composition. Whether these convergence profiles are associated with GC subtype, spatial position, developmental origin or activity history remains to be determined.

Several technical considerations define the current scope of mGRASPi. Stable split-FP complementation makes the framework better suited to structural mapping of synaptic convergence across extended circuit volumes than to monitoring rapid, minute-scale synaptic dynamics *in vivo*. Reversible reporters such as ddFPs offer a complementary approach to transient contacts, although their lower fluorescence intensity currently favors local, high-resolution imaging[53]. The destabilization module was designed to limit the persistence of reconstituted signals, but its effective temporal window remains to be established experimentally. Antibody-based decoding also introduces a practical constraint through depth-dependent signal attenuation in volumetric datasets, although advances in tissue clearing and whole-brain immunostaining may extend multiplex convergence mapping across larger tissue volumes[54]. At the same time, the modular design of mGRASPi broadens the framework beyond combinatorial encoding. Cre-, Flp- and tTA-dependent configurations enable the presynaptic and postsynaptic neuronal populations to be genetically specified independently (**Table 1**). Pre-mGRASPi variants carrying self-cleaving 2A-linked Cre further provide a route for anterograde trans-synaptic Cre transfer[55] and genetic access to postsynaptic neurons defined by their inputs. Together, these configurations extend the

framework from synapse mapping toward genetic access to circuit elements defined by both cell identity and connectivity.

Future studies combining multiplexed mGRASPi with cell-type-specific targeting, physiological recording or activity-dependent labeling could relate synaptic input composition to neuronal response properties and behavior. Because presynaptic identities are decoded post hoc through epitope staining, the framework is compatible with *in vivo* imaging of genetically encoded calcium or neurotransmitter sensors, linking functional responses to the subsequent anatomical decoding of convergent inputs. Input-specific perturbation could test the causal roles of individual afferent populations, while these anatomical measurements could constrain computational models of dendritic integration and circuit computation. Together, multiplexed mGRASPi and SynScope make convergent input architecture accessible as a measurable, cell-resolved feature of neural circuits.

Methods

Reagent and code availability

pre-mGRASPi and post-mGRASP expression vectors, including combinatorial epitope variants, 2A-linked constructs, and Cre-, Flp-, and tTA-dependent AAV configurations, together with detailed plasmid maps and sequences, are available through Addgene (**Table 1**). SynScope source code and documentation are available at GitHub (https://github.com/jinnyLab/SynScope).

Molecular Cloning and Viral Vector Preparation

pre-mGRASPi constructs were engineered as a modular chimeric presynaptic carrier optimized for multiplex synapse labeling and distal axonal targeting. Constructs incorporated a mutant VAMP2 backbone (Δ61–80, M46A) containing the Nx+10 PDZ-binding motif and a PEST destabilization domain, together with split-GFP11 and combinatorial epitope modules for multiplex presynaptic identity encoding. Single- and quadruple-epitope pre-mGRASPi variants containing Myc, V5, HA and Ollas modules were synthesized commercially (ATUM). Restriction enzyme sites (XhoI and EcoRI/NotI) were incorporated upstream of Nx+10 or split-GFP11 within quadruple-epitope construct to enable modular insertion or removal of individual epitope modules. Double- and triple-epitope configurations were subsequently generated through restriction enzyme–based subcloning into pre-existing pre-mGRASPi vectors. These pre-mGRASPi variants (**Table 1**) were subcloned into pAAV-CAG expression vectors (Addgene #34910) using standard molecular cloning procedures. Additional modular variants incorporating self-cleaving 2A-linked iCre recombinase, or mTagBFP were generated using sequence derived from previously described plasmids (iCre was generated in-house[35] and is available from Addgene (Addgene plasmid # 51904 ; http://n2t.net/addgene:51904 ; RRID:Addgene_51904) and pBAD-mTagBFP2 was a gift from Vladislav Verkhusha (Addgene plasmid # 34632 ; http://n2t.net/addgene:34632 ; RRID:Addgene_34632)). Cre-, Flp-, and tTA-dependent AAV configurations were generated using genetic switch-dependent vector backbones derived from previously described plasmids (Cre-dependent vector, pAAV-CAG-JxON, was generated in-house[28] and is available

from Addgene (Addgene plasmid # 34913 ; http://n2t.net/addgene:34913 ; RRID:Addgene_34913). pAAV-Ef1a-fDIO EYFP was a gift from Karl Deisseroth (Addgene plasmid # 55641 ; http://n2t.net/addgene:55641 ; RRID:Addgene_55641) and pAAV-TRE-tdTomato-WPRE was a gift from Takeshi Imai (Addgene plasmid # 104112 ; http://n2t.net/addgene:104112 ; RRID:Addgene_104112)). Structural accessibility and spatial arrangement of appended epitopes relative to Nx+10 and split-GFP11 were evaluated using AlphaFold2 implemented through ColabFold[56]. Predicted structures were visually inspected for potential steric interference and extracellular accessibility of appended epitopes. All constructs were amplified in Stbl2 cells (Thermo Fisher Scientific), and purified using EndoFree Maxi Kits (Qiagen). AAV2/9 particles were packaged by the University of Pennsylvania or University of North Carolina vector cores (1×10^{12}–5×10^{13} vg ml^{-1}).

Animal care and use statement

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology (KIST-IACUC-2024-065). Experimental procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and all applicable institutional and national regulations for animal research.

Adult male and female C57BL/6J mice (12 weeks old; Daehan Biolink, Korea) were group-housed under a 12-h light/dark cycle at controlled temperature (20–23 °C) with ad libitum access to food and water.

Stereotaxic Injections

Mice were anesthetized with isoflurane and stereotaxic injections were performed as previously described in Feng et al[51]. For most experiments, viruses were delivered by pressure injection using pulled glass pipettes (36 μm tip diameter, 45° tip angle) connected to a Nanoliter 2010 injector and Micro-4 syringe pump (World Precision Instruments) at 40 nL/min. For spatially confined labeling of DG GCs for 13-color mossy fiber experiment (**Fig. 1**), iontophoretic viral delivery was performed using glass pipettes (20 μm tip diameter, 45° tip angle) connected via silver wire electrodes (World Precision Instruments, AGW1030) to an electroporation device (Stoelting, #51595). Positive current pulses (5 μA, 7 s intervals) were applied for 7 min, followed by low-current withdrawal (1 μA) to minimize viral leakage. Injection coordinates and virus details are shown in **Supplementary Table 1**. Ophthalmic ointment was applied to prevent corneal drying, and ketoprofen (5 mg kg^{-1}, s.c.) was administered for postoperative analgesia. Mice were maintained in their home cages for at least 2 weeks before tissue collection.

Immunostaining

Brains were collected following transcardial perfusion with 1× PBS, followed by fixation using 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Collected brains were post-fixed overnight at 4 °C. After three washes in 1× PBS for 20 min each, fixed brains were embedded in 3% agarose dissolved in 1× PBS and sectioned at 50–100 μm thickness using a vibratome (Leica VT1200S) at a cutting speed of 0.4 mm s^{-1}. Throughout immunohistochemistry (IHC) procedures, brain sections were maintained in TBST to facilitate antibody penetration. Brain slices were permeabilized at room temperature (RT) for 1 h and blocked with 5% normal goat serum (NGS), 5% bovine serum albumin (BSA), and 2% Fab fragment donkey anti-mouse IgG containing 1% TBST for 1 h. Sections were then incubated with primary antibodies (in 3% NGS,

1% TBST) for 3 days at 4 °C on a shaker. After three 20-min washes, slices were incubated with secondary antibodies (in 2% NGS, 1% TBST) overnight at 4 °C on a shaker. For co-labeling of epitopes and neurotransmitter-specific vesicular transporters, brains were sectioned at 50 μm thickness. Brain sections were permeabilized and blocked as described above, followed by incubation with primary antibodies (3% NGS, 0.4% TBST) overnight at 4 °C. After three washes in 0.1% TBST, sections were incubated with secondary antibodies (2% NGS, 0.1% TBST) for 2h at RT. Prior to mounting, brain sections were then washed once for 20 min in 1 or 0.1% TBST and twice for 20 min each in TBS. Sections were briefly equilibrated in 10 mM Tris-HCl and mounted using Anti-fade Mounting Medium (Vector Laboratories H-1400) after partial drying of tissue edges. All antibody details and dilutions are shown in **Supplementary Table 2**.

Tissue expansion

For expansion microscopy, synapses were labeled by injecting pre- and post-mGRASP viruses into CA3 and contralateral CA1, respectively. Brains were collected and sectioned into 100-μm sections as described above. To verify the localization of reconstituted mGRASPi signals at the synaptic cleft, we indirectly immunolabeled the endogenous presynaptic marker Bassoon and the postsynaptic marker Homer1. After staining, brain tissues were expanded following the protocol described in Tillberg et al.[48]. Briefly, brain slices were incubated in AcX solution (Acryloyl-X, SE; 6-((acryloyl)amino)hexanoic acid, succinimidyl ester; 0.1 mg/mL in 1× PBS) for 1 h at RT, followed by three washes with 1× PBS for 20 min each. The slices were then incubated in gelation monomer solution prepared in 1× PBS containing 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N′-methylenebisacrylamide, 0.01% 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl, 0.2% (w/w) ammonium persulfate, and 0.2% (w/v) tetramethylethylenediamine at 4 °C for 30 min. Gelation was induced by incubating the slices in a humidified chamber at 37 °C for 2 h. The gels were then digested with Proteinase K (8 U/mL) in digestion buffer containing 50 mM Tris, 1 mM EDTA, and 0.5% (w/w) Triton X-100, pH 8.0, at 37 °C for 4 h. After digestion, the gels were transferred to 90-mm cell culture dishes and washed with fresh 1× PBS 4–6 times at RT until the samples expanded approximately two-fold relative to their original size. Details of materials are provided in **Supplementary Table 2.**

Image acquisition

Wide-field images were acquired using an Axioscan Z1 slide scanner (Carl Zeiss Microscopy) equipped with a 10× 0.45 NA Plan-Apochromat air lens. Confocal images were acquired using an LSM 980 microscope equipped with Airyscan2 (Carl Zeiss Microscopy). Imaging was performed with 40× 1.4 NA Plan-Apochromat oil-immersion lens with 2× digital zoom, yielding an image resolution of 0.076 μm/pixel. For z-stack imaging, optical sections were collected at 0.5 μm intervals. Blue fluorophores (Alexa Fluor 405; AF405), green fluorophores (AF488, sfGFP, and AF514), red fluorophores (AF555 and dTomato), and far-red fluorophores (AF633) were excited using 405 nm, 488 nm, 561 nm, and 633 nm lasers, respectively. Emission signals were detected at 389–550 nm for AF405, 469–677 nm for AF488 or sfGFP, 570–726 nm for AF555 and dTomato, and 608–803 nm for AF633. To distinguish reconstituted sfGFP signals from AF514-labeled axonal signals within the green spectral range, emission windows were spectrally separated during acquisition. sfGFP signals were collected at 469–517 nm, whereas AF514 signals were collected at 546–677 nm to minimize

overlap between synaptic puncta and axonal labeling (**Fig. 2f**). Tile stitching and Airyscan processing were performed using ZEN blue software (Carl Zeiss Microscopy). Images of expanded brain slices were acquired using a Nikon Eclipse Ti2-E microscope equipped with a Dragonfly 200 spinning disk confocal system (Andor, Oxford Instruments, Abingdon, UK) and a Zyla 4.2 sCMOS camera (Andor, Oxford Instruments). Imaging was performed with a 40×, 1.15 NA water-immersion objective.

Image Preprocessing

Shading correction. A custom Python implementation of the BaSiC algorithm[57-59] was used for flat-field estimation and illumination normalization. Multi-tile stitched images acquired using ZEN blue software (Carl Zeiss Microscopy) were separated into individual imaging tiles and downsampled by 2 × 2. Flat-field illumination profiles were estimated from 20 randomly sampled z-frames per tile using default parameters and applied to each fluorescence channel after resizing to the original tile dimensions. Corrected tiles were subsequently restitched into volumetric image stacks using custom Python script.

Chromatic shift correction. Chromatic misalignment was corrected using a custom registration pipeline implemented in ANTsPy[60]. Designated channels were aligned to a fixed reference channel using symmetric normalization with normalized cross-correlation (SyNCC), enabling alignment across both lateral (XY) and axial (Z) dimensions.

Z-signal correction. A modified ISCL framework[49] was used to correct depth-dependent signal attenuation. Histogram matching was first applied to each z-frame using a single user-defined reference frame to normalize frame-by-frame intensity distributions. The workflow additionally required matched clean and noisy frames selected from high signal-to-noise and signal-attenuated imaging depths, respectively; the reference frame was typically chosen as the second-brightest z-frame. The ISCL model was trained on multi-tile image datasets for 400 iterations using two NVIDIA TITAN X GPUs (batch size = 16; learning rate = 0.0001), with hyperparameters optimized using Fréchet Inception Distance[61] (FID) and Learned Perceptual Image Patch Similarity[62] (LPIPS) metrics. A pretrained ISCL model is provided with the SynScope package, which additionally supports retraining using user-supplied datasets.

Dendrite subtraction. Thickness-based morphological segmentation (`skimage.measure.regionprops`) was used for dendrite subtraction. For each connected component, thickness was defined as component area divided by major axis length. Components exceeding a predefined thickness threshold (≥5 pixels) were classified as dendritic structures and removed from thresholded images. Remaining signals were refined using morphological dilation (`skimage.morphology.dilation`; structuring element = 3 × 3 pixels).

Synapse-centered binary decoding

Synapse detection. mGRASPi puncta were detected using the mGRASPi puncta detection package[50,51] and manually proofread. Detected puncta were stored as 3D voxel clusters (`.nimp`).

Adaptive thresholding. For each detected synapse, punctum-centered ROIs were extracted from three consecutive frames (z−1, z, z+1) using the corresponding x, y, and z coordinates. Pixel intensity distributions

within small ROIs (10 × 10 pixels) were modeled for each fluorescence channel using a two-component GMM (`sklearn.mixture.GaussianMixture`), and thresholds were defined as the midpoint between component means. Thresholded images were used to generate synapse-centered signal masks for downstream decoding.

Rule feature extraction. Rule vectors were constructed from synapse-centered binary masks to encode overlap, morphology, and inter-channel relationships. Overlap features were quantified using an overlap metric defined as 0.5 × Jaccard index + 0.5 × dice coefficient. Morphology features included skeleton length, eccentricity, and orientation. Graph features represented channels as nodes and pairwise overlaps as edges, with connections defined by overlap metric ≥0.60. Graph connectivity and clique structure captured higher-order co-localization patterns. Features were concatenated into fixed-length rule vectors for each punctum.

Synapse classification

Framework development and model training. For development of the synapse classification framework, ground-truth synapse labels were generated by expert annotation of detected mGRASPi puncta (total n = 29,033; 4-input DG, n = 25,055; 13-input mPFC, n = 3,978). Initial framework optimization was performed using a 4-input DG dataset consisting of 4 image tiles (n = 24,090), including establishment of overlap-, morphology-, and graph-based rule features together with optimization of a hybrid rule-based and supervised learning framework for synapse classification. For final model training, rule vectors derived from overlap-, morphology-, and graph-based features were used to train a binary LightGBM[52] classifier for channel co-localization prediction. The final training dataset consisted of 2,803 labeled ground-truth synapses collected across multiple 13-input datasets. Of these, 1,395 ground-truth synapses were divided into training (80%) and held-out test (20%) subsets using stratified sampling, whereas 1,408 ground-truth synapses were used for optimization and calibration. To address class imbalance, balanced per-sample weights and SMOTE-based oversampling were applied to the training subset. Training was performed on an NVIDIA Tesla V100 GPU for up to 1,000 boosting rounds with early stopping based on validation log-loss. Decision thresholds were calibrated by maximizing F1 score on the held-out test set. The classifier achieved an accuracy of 0.925, AUC–ROC of 0.970, F1 score of 0.892, precision of 0.875, and recall of 0.911.

Synapse classification. Pairwise channel co-localization probabilities predicted by the LightGBM classifier were binarized to define edges within an undirected channel graph, and the maximum clique was selected as the predicted channel combination. Mean edge probability defined the confidence score, and biological constraints were applied to reject invalid assignments and enforce consistency with mGRASPi labeling logic.

Validation. The finalized framework and classifier pipeline were evaluated on an independent DG image tile selected by expert annotation to contain balanced representation of presynaptic inputs (965 ground-truth synapses), and on an independent 13-input mPFC dataset containing 1,175 ground-truth synapses for final evaluation.

Web-based interface

A web-based SynScope platform was implemented using a node-based visual workflow architecture adapted from the work of Jeon et al.[63], enabling modular configuration of preprocessing, synapse detection, and interactive inspection of synapse assignments. Preprocessing, synapse detection, and synapse assignment modules were implemented as configurable nodes based on custom Python code within a visual workflow interface, with user-adjustable settings provided for each node (**Table 2**).

Synapse quantification and analysis

Synapse quantification and subsequent analyses were performed using custom Python scripts. Typed mGRASP puncta were loaded from `.nimp` files, grouped by presynaptic identity, and converted to physical coordinates using voxel dimensions from image metadata or puncta-detection logs.

Four-input DG analysis and morphological reconstruction. A cohort of 125 GCs was analyzed for four presynaptic inputs: MCs, MEC, LEC and SuM. All GCs were reconstructed using neuTube[64] and exported as `.swc` files. Puncta were assigned to dendritic arbors by nearest-node mapping, with a maximum node-to-punctum distance of 0.6 μm. Dendritic arbor length was calculated as the sum of Euclidean lengths of all parent–child segments, and input-specific dendritic density was expressed as puncta per unit dendritic length (puncta μm^{-1}).

The soma was identified as the SWC node with the largest radius. Its surface area was estimated assuming a spherical geometry $A_{soma} = 4\pi r^2$, after converting the radius to micrometres using the mean in-plane voxel size. SuM puncta were detected separately within dendritic and soma-associated compartments. Dendritic SuM density was normalized to dendritic arbor length, whereas soma-associated SuM density was normalized to estimated soma surface area (puncta μm^{-2}). Unless otherwise specified, both compartments were included in SuM input-composition analyses.

Laminar boundary detection and punctum assignment. Molecular-layer boundaries were defined from afferent-marker distributions in the pre-mGRASPi channels. The blue channel identified the foreground containing OML and MML, whereas the yellow channel contained IML. For each confocal z-plane, the blue channel was Gaussian-smoothed ($\sigma \approx 2$ pixels) and thresholded using Otsu's method with a lower percentile constraint. The resulting mask underwent morphological opening and closing, hole filling, and removal of small connected components. The retained molecular-layer component defined the outer-ML and GCL–IML boundaries through its superficial and deep edges, respectively.

The IML-associated signal was identified from the yellow channel using a background-suppressed score $S = clip(I_{blue} - 0.55I_{yellow}, 0),$ followed by Gaussian smoothing and thresholding. The IML–MML boundary was derived from this signal, and the MML–OML boundary was placed midway between the IML–MML and outer-ML boundaries. Boundary detection was performed on a 2× downsampled grid. Missing coordinates and z-planes were linearly interpolated, and boundaries were regularized by outlier clipping and spatial smoothing (σ = 15–40 pixels along *x* and σ = 1.5 slices along *z*) before interpolation to the full-resolution grid.

The GCL was delimited by the GCL–IML boundary and a hilus–GCL boundary defined by the deep edge of GCL-associated fluorescence. Automatically detected boundaries were visually inspected across the z-stack, and individual z-slice boundaries were manually adjusted when needed to correct anatomical misalignment. Soma depth was calculated as the fractional distance from the GCL–IML boundary toward the hilus–GCL boundary at the soma's (*x*, *z*) position and classified as superficial (0–1/3), middle (1/3–2/3), or deep (2/3–1) GCL. Each GC was assigned to the upper or lower DG blade according to its dataset of origin. Puncta were assigned to IML, MML or OML based on their positions relative to the corresponding boundaries on the nearest z-plane.

Input composition, correlations and PCA. For each GC, the fractional contribution of each input was calculated as shown in

$$f_i = \frac{N_i}{\sum_j N_j},$$

where N_i is the number of puncta from input i. The denominator comprised all typed MCs, MEC, LEC and SuM puncta, including both dendritic and soma-associated SuM puncta. GCs were ordered by descending MCs fraction for heatmap and population-profile visualization. For stacked profiles, input fractions were smoothed using a nine-cell moving average and renormalized to sum to one at each cell rank.

Pairwise relationships between input-specific dendritic densities and input-composition fractions were assessed using Pearson correlation, with two-sided tests of zero correlation. Partial Spearman correlations were calculated between ranked input-specific dendritic densities while controlling for ranked total dendritic synapse density, defined as the total number of typed dendritic puncta divided by dendritic arbor length.

For principal component analysis (PCA), a pseudocount of 0.5 was added to each entry of the GC-by-input count matrix (MCs, MEC, LEC and SuM), and each row was normalized to sum to one. Centered log-ratio (CLR) coordinates were calculated by subtracting the mean log-count across the four inputs within each GC from each log-count. Each input column was then mean-centered across GCs, and PCA was performed by singular value decomposition without scaling by standard deviation. Loadings for the first principal component (PC1) were extracted from this covariance-based PCA.

Input-ratio null-model analysis. For each GC, log-transformed LEC-to-MCs and LEC-to-SuM count ratios were calculated as

$$R_{ref} = log\left(\frac{N_{LEC} + 0.5}{N_{ref} + 0.5}\right),$$

where N_{ref} denotes either MCs or SuM counts. Null distributions were generated by multinomial resampling from the pooled input composition across all GCs while preserving each GC's observed total synapse count. Observed ratio distributions were compared with their corresponding null distributions.

13-input mPFC analysis. For the 13-input medial prefrontal cortex (mPFC) dataset, puncta were grouped into 13 presynaptic input classes. Cortical layer assignment was performed independently of dendritic

morphology using anatomical depth ranges: layer 1, 0–170 μm; layers 2/3, 170–300 μm; layer 5, 350–550 μm; and layer 6, 600–850 μm[65]. Layer-specific input composition was calculated as the proportion of typed puncta contributed by each input class within each layer.

Statistical analysis. The individual GC was the unit of analysis for population-level comparisons and correlation analyses. Statistical analyses were performed in Python using NumPy, pandas and SciPy. Group comparisons were performed using two-sided Mann–Whitney U tests. Correlations were assessed using two-sided tests of the null hypothesis of zero correlation. Multiple pairwise comparisons were corrected using the Benjamini–Hochberg false-discovery rate procedure. Data are presented as mean ±s.e.m. unless otherwise indicated, with $P < 0.05$ considered significant.

Supplementary Movies

Narration for Supplementary Movies was generated using the AI-based text-to-speech platform ElevenLabs (accessed May 2026). The generated voice-over was used solely for explanatory narration of the movies. All narration scripts were written, reviewed, and approved by the authors. ElevenLabs was not used for data analysis, data interpretation, figure generation, or scientific writing of the manuscript.

Data Availability

The raw microscopy datasets and processed data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

Jinhyun Kim conceived and supervised the study and designed the pre-mGRASPi components. Jihyun Kim implemented and validated experimental strategies, conducted experiments and data acquisition, and generated ground-truth datasets for synapse analysis and identity decoding. Y.K.C. developed the SynScope framework and implemented preprocessing, synapse-classification, combinatorial decoding, and analysis pipelines and conducted the analysis. H.J. contributed to development of the z-signal correction framework. S.J. implemented the web-based user interface platform. W.K.J. provided guidance on the z-signal correction framework, synapse-classification methods, and web-based interface implementation. C.E.P. and J.B.C. performed expansion microscopy experiments. Jihyun Kim, Y.K.C., and Jinhyun Kim wrote the original draft of the manuscript. All authors reviewed and edited the manuscript.

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Figure legends

Figure 1 | Design and multiplex implementation of mGRASPi for scalable synaptic convergence mapping

a. Schematic overview of multiplexed mGRASPi. Synaptic contacts are detected through reconstitution of a single split-GFP pair, while distinct presynaptic populations are independently represented by combinatorial epitope-defined color identities, enabling scalable visualization of convergent synaptic inputs onto single neurons.

b. Combinatorial encoding scheme for multiplexed input discrimination. Binary combinations of 4 epitope channels generate distinct presynaptic identities while preserving dedicated channels for synapse detection and postsynaptic labeling. 13 combinatorial epitope identities assigned to individual presynaptic inputs are shown on the right.

c. Modular design of pre-mGRASPi. The construct incorporates an engineered VAMP2-based presynaptic carrier (eng.SNARE), split-GFP11 (spG11), combinatorial epitope modules (E(x1–x4)), and optional 2A-linked effector genes. Epitope modules can be flexibly combined upstream of the carrier or split-GFP11 to generate distinct presynaptic identities. SP, signal peptide; DD, destabilization domain; TM, transmembrane domain; Nx+10, Neurexin-1β–derived PDZ-binding motif; GS linker, glycine-serine linker.

d-f. Synaptic detection of mGRASPi in the hippocampal CA3–CA1 circuit. AAVs expressing pre-mGRASPi(V5) and post-mGRASP were delivered into CA3 and CA1, respectively. High-magnification confocal images (**d, left**) and high-resolution 3D Airyscan rendering (**e**) show discrete reconstituted mGRASPi puncta at sites of intersection between blue CA3 axons and red CA1 dendritic spines. Expansion microscopy images (**f**) show reconstituted puncta localized between immunostained presynaptic Bassoon and postsynaptic Homer signals. Scale bars, 1 mm (d, left), 1 μm (d, right).

g–i. Multiplexed labeling of DG granule cell mossy fiber projections using combinatorial pre-mGRASPi variants. 13 combinatorial pre-mGRASPi variants were delivered to 13 spatially separated sites along the GCL (**g**). Brain slices were immunostained using epitope-fluorophore combinations consisting of V5- Fluor (AF) 405, HA-AF488, Myc-AF555, and Ollas-AF633. 4-channel merged images in the CA3 stratum lucidum (**h**) displayed intermingled and heterogeneous multicolored mossy fiber projections distinguished by combinatorial epitope labeling. Representative images were acquired at 0.2 mm intervals along the anterior–posterior (AP) axis from bregma −1.6 mm to −2.8 mm. Cropped higher-magnification images (**i**) show representative individual mossy fiber boutons resolved into 13 distinct combinatorial epitope identities. ML, mediolateral; DV, dorsoventral. so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; sl, stratum lucidum. Scale bars, 200 μm (h top), 100 μm (h bottom left), 5 μm (h bottom right), 1 μm (i).

Figure 2 | Multiplexed synaptic convergence mapping in multi-input circuits using mGRASPi

a. Schematic illustration of multiplexed convergence mapping in DG. Distinct AAVs expressing pre-mGRASPi variants were injected in the LEC, MEC, SuM, and MCs, with corresponding combinatorial epitope identity as indicated schematically on the right.

b. Distinct presynaptic populations labeled with combinatorial epitopes were detected within characteristic laminar domains of the DG molecular layer, consistent with known projection patterns of LEC, MEC, MCs, and SuM inputs. Brain sections were immunostained using epitope–fluorophore combinations consisting of V5–alexa fluor (AF)405, Myc–AF555, and Ollas–AF633. Higher-magnification image (right) shows layer-specific convergence of combinatorially labeled axons onto a superimposed reconstructed DG granule cell dendritic arbor shown in white, reconstructed using neuTube[64].

Scale bars, 200μm (left), 20μm (right).

c. Representative synaptic contacts corresponding to distinct presynaptic inputs identified by combinatorial epitope-defined identities, corresponding to regions 1–4 indicated in the higher-magnification image in **b**. Individual mGRASPi puncta were resolved at sites of contact between red dendritic spines and presynaptic boutons originating from LEC, MEC, MCs, and SuM inputs. Orthogonal 3D views (right) illustrate representative synaptic configurations corresponding to each decoded presynaptic identity. Images were gamma-corrected for visualization. Scale bars, 1 μm (merged images).

d,e. Experimental design for 13-input convergence mapping onto mPFC. Post-mGRASP was expressed in mPFC neurons, while 13 anatomically and neurotransmitter-distinct presynaptic populations were labeled using combinatorial epitope-tagged pre-mGRASPi variants. Sagittal brain atlas schematics show glutamatergic cortical and thalamic inputs (contra. ACC, contra. PL, contra. IL, AI, MD, VM, BLAd, BLAv, and vCA1), dopaminergic inputs (VTAm and VTAl), serotonergic input (DR), and cholinergic input (BF) converging onto mPFC. Corresponding AAVs expressing combinatorial pre-mGRASPi identity codes injected to each presynaptic population are shown on **e**. Epitope–channel combinations were selected based on projection characteristics such as length and density (**Extended Data Fig. 5**).

f. Multiplexed visualization of 13 convergent presynaptic projections onto neurons in the prelimbic (PL) region of the mPFC (top left), together with a representative reconstructed layer 5 (L5) pyramidal neuron and its detected synaptic contacts (bottom left). Intermingled combinatorially labeled axonal projections from 13 presynaptic populations were detected using epitope–fluorophore combinations consisting of HA–AF405, V5–AF514, Myc–AF555, and Ollas–AF633. Despite dense overlap of presynaptic axons, cropped high-magnification images of individual synaptic contacts show discrete resolution and decoding of distinct presynaptic populations based on combinatorial epitope identities (right). Individual mGRASPi puncta were detected at sites of contact between red dendritic structures and combinatorially labeled presynaptic boutons, enabling synapse-level identification of convergent inputs. Images were gamma-corrected for visualization. L, layer. Scale bars, 100 μm (left), 1 μm (right).

Fig. 3 | SynScope pipeline for multiplex synaptic convergence decoding

a. Overview of the SynScope workflow for synapse-level decoding of multiplexed convergent inputs. Multiplexed mGRASPi datasets containing reconstructed neurons, detected synapses, and raw multi-channel fluorescence images were sequentially processed through preprocessing, synapse-centered binary decoding, and synapse assignment to generate decoded convergence maps with presynaptic identity labels.

b. Shading correction for normalization of illumination and tile-stitching artifacts. Schematic illustrations of optical illumination inhomogeneity and tile-dependent intensity variation are shown at the top. Representative images before and after correction are shown below.

c. Chromatic shift correction for cross-channel image alignment. Lateral (XY) and axial (XZ) chromatic misalignments between imaging channels (ch1 and ch4), illustrated at the top, were corrected using sub-micron registration. Representative axonal boutons before and after correction are shown below.

d. Z-signal correction for depth-dependent signal attenuation. Uneven antibody and light penetration across imaging depth (left) were compensated using z-dependent signal normalization. Representative image slices before and after correction across z-planes are shown on the right.

e. Synapse-centered adaptive thresholding and binary decoding. Local ROIs were generated around detected synaptic puncta, and Gaussian mixture model (GMM)-based adaptive thresholding was applied within each ROI to generate binary axon masks corresponding to individual imaging channels.

f. Extraction of overlap- and morphology-based biological rules from synapse-centered binary masks. Overlap metrics, orientation consistency, eccentricity, and axonal continuity were used to distinguish coherent axonal structures from incidental overlap between unrelated axons (**Extended Data Fig. 7b**). Representative examples illustrating rule-based feature extraction are shown on the right. In synapse 1, overlap-based analysis identified co-localization between ch1 and ch2, resulting in decoding as (1+2). In synapse 2, overlapping signals were further resolved using orientation consistency, leading to decoding as (1+3). In synapse 3, axonal continuity across adjacent regions was used to distinguish coherent axonal structures from incidental overlap.

g. Graph-based channel association for combinatorial identity decoding. Inter-channel relationships were represented as graphs in which nodes denote detected channels and edges represent overlap-based co-localization between axons.

h. Synapse classification framework for presynaptic identity assignment. Features derived from overlap-, morphology-, and graph-based biological rules were integrated using LightGBM for final synapse classification and presynaptic identity assignment.

i. Quantitative evaluation of SynScope decoding performance and ablation analysis. Removal of individual preprocessing modules demonstrated that shading correction, chromatic shift correction, and z-signal correction each substantially contributed to multiplex decoding performance (left). Synapse-centered adaptive thresholding additionally outperformed global thresholding approaches (right). Combined use of the complete preprocessing pipeline and local adaptive thresholding enabled robust presynaptic identity assignment across 2,803 ground-truth synapses from multiple 13-input datasets, achieving 91.6% decoding accuracy (F1 score = 0.855).

Fig. 4 | Layer-specific multi-input synaptic convergence revealed by mGRASPi and SynScope

a. Representative reconstructed mPFC L5 pyramidal neuron with synaptic contacts assigned to 13 distinct presynaptic populations based on decoded combinatorial epitope identities from **Fig. 2**. Colored puncta indicate SynScope-assigned synapses originating from individual convergent inputs distributed across cortical dendrites. Insets show representative raw 4-channel merged fluorescence images (left) and the corresponding reconstructed dendritic segments with assigned synaptic identities (right), illustrating synaptic convergence along an individual dendritic branch. L, layers. Scale bar, 5 μm.

b. Representative examples of synapse identity decoding from individual mGRASPi puncta shown in the inset of **a**. Synapse-centered local ROIs were subjected to channel-wise adaptive thresholding to generate binary axon masks for individual imaging channels. In the red-spectrum channel (ch3), dendritic morphology-based subtraction was applied before axonal classification, as described in **Extended Data Fig. 7a**. Binary channel associations were then integrated using graph-based combinatorial rules to assign presynaptic identities. Representative processed channel masks, graph-based channel associations, and decoded identities are shown from left to right.

c. Sankey diagram illustrating layer-specific convergence organization across cortical layers in the 13-input mPFC dataset (12,056 decoded synapses). Flow widths represent the relative contribution of distinct presynaptic populations to synaptic inputs within individual cortical layers, highlighting heterogeneous and layer-convergent input organization in the mPFC.

Fig. 5 | Laminar organization and cell-to-cell variability of synaptic inputs to DG GCs

a. Three-dimensional reconstructions of DG GCs (n = 125), traced with neuTube[64] and displayed to a common DG reference mesh. The inset shows the location of the reconstructed GCs within the DG. Anatomical axes are indicated (DV, dorsoventral; AP, anteroposterior; ML, mediolateral).

b. Heat map of input-specific synaptic density across individual GCs. Each row represents a GC, and columns represent inputs from the SuM, MCs, MEC and LEC. Synaptic densities are expressed as z-scores.

c. Pairwise relationships between input-specific synaptic densities across GCs. Scatter plots show the relationships between MCs and MEC (r = 0.66), MCs and LEC (r = 0.37), MEC and LEC (r = 0.72), MEC and SuM (r = 0.14), MCs and SuM (r = 0.40) and LEC and SuM (r = −0.05). Each point represents an individual GC; red lines indicate linear fits, and dashed lines indicate reference levels.

d. Representative GC dendritic reconstructions showing the spatial distribution of mapped synapses from LEC (magenta), MEC (cyan) and MCs (yellow). Synaptic contacts are overlaid on dendritic arbors across the GCL, IML, MML and OML.

e. Layer-specific synaptic density, expressed as the number of synaptic puncta per unit dendritic length (puncta per μm), across the GCL, IML, MML and OML. Stacked bars show the contributions of LEC, MEC, MCs and SuM inputs. Values above the bars indicate mean synaptic densities across cells; error bars indicate s.e.m.

f. Representative examples of synapse identity assignment within the MML. Enlarged image patches show individual synapses assigned to LEC-, MEC-, or MCs-derived inputs together with corresponding binary masks and morphology-aware decoding outputs, highlighting the intermixed convergence of multiple presynaptic populations within the MML. Arrows indicate assigned synapses.

g. Input-specific synaptic density across GCs, expressed as puncta per μm of dendritic length. Bars show mean densities across cells for SuM (0.017), MCs (0.094), MEC (0.089) and LEC (0.135) inputs; error bars indicate s.e.m.

h. Heat map of input composition across GCs, sorted by decreasing MCs fraction. Each row represents an individual GC, and columns represent MCs, MEC, LEC and SuM inputs. Colors indicate the relative composition fraction for each input.

i. Pairwise relationships between input composition fractions across GCs. Scatter plots show MCs versus MEC (r = 0.07), MCs versus LEC (r = −0.89), MEC versus LEC (r = −0.26), MEC versus SuM (r = −0.22), MCs versus SuM (r = 0.32) and LEC versus SuM (r = −0.50). Each point represents an individual GC; red lines indicate linear fits, and dashed lines indicate reference levels.

j. Stacked composition profile of synaptic inputs across GCs ordered by decreasing MCs fraction. Input fractions are smoothed using a nine-cell moving average and renormalized to sum to one at each cell rank. The smoothed fractions are stacked in the order SuM, MCs, MEC and LEC, producing a cumulative composition profile that spans the full composition-fraction range (0–1) at each rank.

k. Principal component 1 (PC1) loadings for the four input populations. Bars show the contribution of each input to PC1, with positive loadings for MCs and SuM and negative loadings for MEC and LEC.

l. Distributions of log-transformed LEC-to-MCs and LEC-to-SuM synapse-count ratios, calculated as $log[(n_{LEC} + 0.5)/(n_{ref} + 0.5)]$, where n denotes synapse count and the reference input is MCs or SuM. For the LEC-to-MCs ratio, the gold curve represents the observed distribution and the dark gray curve the null distribution. For the LEC-to-SuM ratio, the purple curve represents the observed distribution and the light gray curve the null distribution. Null distributions were generated by multinomial resampling, preserving each GC's total synapse count and sampling input identities according to the pooled input composition across all GCs.

Extended Data Figure legends

Extended Data Fig.1 | Efficient axonal trafficking of presynaptic mGRASPi

a. Schematic illustration of the experimental design and representative images comparing axonal trafficking efficiency between mGRASPi and smFP. Schematic illustrations of the experimental design are shown on the left, with corresponding representative projection images shown on the right. To evaluate axonal enrichment of mGRASPi, epitope-matched mGRASPi and smFP constructs were injected into two independent projection systems (hippocampal CA3 and primary motor cortex (MOp)) in a crossover manner across animals (virus titer, 2.5×10^{12} vg mL^{-1}). In the upper panels, smFP (cyan) was expressed in the MOp circuit and mGRASPi (orange) in the CA3 circuit, whereas in the lower panels, mGRASPi (orange) was expressed in the MOp circuit and smFP (cyan) in the CA3 circuit. Brain sections containing representative projection regions were immunostained using V5–AF488 and imaged. Representative MOp projection regions included the contralateral MOp, ipsilateral and contralateral STR, globus pallidus (GP), and thalamus (TH), whereas CA3 projection regions included ipsilateral and contralateral CA1. Colored outlines correspond to projection ROIs used for fluorescence quantification under smFP (cyan) and mGRASPi (orange) conditions.

b. Mean fluorescence intensity was measured from defined projection ROIs using ImageJ and normalized to the corresponding injection-site intensity, defined as 100%. For quantitative comparison, images from both conditions were visualized using identical display intensity ranges. Normalized fluorescence intensity profiles across projection regions are shown as line plots across projection regions for the MOp and CA3 circuits,

enabling comparison of axonal labeling across projection distances. Compared with smFP, mGRASPi exhibited 1.2–6.5-fold higher fluorescence intensity across projection regions. The difference became more pronounced at increasingly distal projection sites. mGRASPi additionally showed more uniform fluorescence distribution across long-range projections, consistent with efficient trafficking and enrichment of pre-mGRASPi components throughout distal axons and presynaptic terminals.

Extended Data Fig. 2 | mGRASPi detects synapses across diverse neurotransmitter-defined circuits

a. Schematic overview of 4 neurotransmitter-defined circuits used to evaluate the applicability of pre-mGRASPi across diverse circuit types, including contralateral mPFC→mPFC glutamatergic projections (black), ipsilateral GPe → striatum (STR) GABAergic projections (black dashed), ipsilateral SNc→STR dopaminergic projections (green), and DR → BLA serotonergic projections (violet) are shown at the top. Representative 4-channel merged overview images (left) together with magnified merged and channel-separated images (right) are shown for each circuit. Magnified images correspond to regions indicated by white boxes in the overview images. Reconstituted mGRASPi puncta indicated by arrowheads were detected along postsynaptic dendritic structures and aligned with presynaptic axons labeled with V5 or Ollas together with corresponding neurotransmitter-associated axon terminal markers. Brain sections were immunostained using antigen–fluorophore combinations consisting of V5–AF405, Ollas–AF633, vesicular glutamate transporter 1 (vGLUT1)–AF633, vesicular GABA transporter (vGAT)–AF405, vesicular dopamine transporter (DAT)–AF633, and vesicular serotonin transporter (SERT)–AF633. Scale bars, 10 μm (overview images) and 1 μm (magnified merged images).

Extended Data Fig. 3 | Synapse labeling across 27 distinct neural circuits

a. Synapse labeling across 27 distinct circuits spanning hippocampal-, cortex-, and basal ganglia-related networks. Schematic illustrations of representative circuit architectures are shown at the top, with corresponding mGRASPi imaging data shown below. Representative examples include DG convergence circuits (SuM, MCs, MEC, LEC → DG), hippocampal projection pathways (DG → CA3, SuM → CA2, ipsilateral and contralateral CA3 → CA1), mPFC convergence circuits (contra. ACC, contra. PL, contra. IL, AI, MD, VM, BLAd and BLAv, vCA1, VTAm, VTAl, DR, and BF → mPFC), and basal ganglia-related circuits including subthalamic nucleus (STN) convergence circuit (MOp and GPe → STN), SNc → STR, VTA → Nucleus accumbens (NAc), and Gpe → STR. Reconstituted mGRASPi puncta were consistently detected at sites of apposition between presynaptic axons and postsynaptic structures across diverse circuits. Insets show representative higher-magnification views of mGRASPi-labeled synaptic contacts from individual circuits. In magnified images, presynaptic axons are shown in blue (except for mPFC synaptic panels), postsynaptic structures in red, and reconstituted mGRASPi puncta as green. Scale bars, 100 μm (overview images), 10 μm (magnified images), and 1 μm (mPFC synapse panels).

Extended Data Fig. 4 | Imaging performance of epitope–fluorophore channel combinations

a. Quantitative evaluation of signal-to-noise ratio (SNR) across epitope–channel combinations for multiplexed mGRASPi imaging. Single-epitope pre-mGRASPi variants expressing V5, Ollas, HA, or Myc were injected

into the hippocampal CA3 region (virus titer, 1.1×10^{12} vg mL^{-1}). Brain sections were immunostained using predefined epitope–fluorophore combinations consisting of AF405, AF488, AF555, and AF633 secondary antibodies. Columns indicate individual epitopes (V5, Ollas, HA, and Myc), whereas rows indicate fluorescence channels corresponding to AF405, AF488, AF555, and AF633. All images within each fluorescence channel were acquired and visualized using identical imaging and display settings. Representative signal and background ROIs used for SNR quantification are indicated by magenta and yellow boxes, respectively (left top). SNR values displayed within images indicate calculated signal-to-noise ratios for each epitope–channel combination, calculated as shown below. Quantitative evaluation of SNR and depth-dependent signal attenuation was used to guide practical selection of epitope–channel configurations for multiplexed convergence mapping.

b. Evaluation of depth-dependent fluorescence attenuation and antibody penetration across epitope–channel combinations. Representative orthogonal side views of volumetric z stack images are shown before (raw,left) and after z-signal correction (z-correction, right, see **Extended Data Fig. 6**) for each epitope–channel combination. Columns indicate individual epitopes (V5, Ollas, HA, and Myc), whereas rows indicate fluorescence channels corresponding to AF405, AF488, AF555, and AF633. Orthogonal views were generated from 100 μm-thick tissues and volumetric image stacks were acquired at 0.5 μm z-intervals using confocal microscopy (LSM 980, 40x objective). Corresponding mean fluorescence intensity profiles across imaging depth are shown below. Solid lines indicate raw fluorescence signals, whereas dotted lines indicate z-corrected signals. Plot colors correspond to fluorescence channels shown on the left (blue, AF405; green, AF488; red, AF555; magenta, AF633). Although z-signal correction substantially restored fluorescence intensity across all combinations, epitope–channel combinations with higher antibody penetration and SNR (**a**) remained preferable for reliable multiplex detection.

Extended Data Fig. 5 | Design guidelines for epitope–fluorophore channel selection

a. Input number–dependent epitope assignment schemes for multiplexed pre-mGRASPi labeling. Representative combinatorial epitope assignment strategies are shown for configurations containing 1–14 convergent presynaptic inputs. Colored labels indicate assigned epitope identities and corresponding fluorescences (blue, AF405; yellow, AF514; red, AF555; magenta, AF633). For configurations containing six or fewer inputs, the green channel was reserved exclusively for mGRASPi detection, enabling direct visualization of reconstituted synaptic puncta without spectral separation. For seven or more inputs, incorporation of AF514-based axonal labeling into the green spectral range expanded combinatorial coding capacity and required spectral separation between AF514-labeled axonal signals and mGRASPi signals. Across all configurations, single-channel red (AF555) assignments were excluded to minimize ambiguity with postsynaptic dTomato labeling. Additional multiplex expansion can be achieved through increased combinatorial epitope complexity and expanded use of spectrally separated imaging channels.

b. Practical considerations for epitope assignment based on projection properties and multiplex imaging conditions. For long-range distal projections, V5 and Ollas are recommended because of their efficient immunolabeling characteristics (see **Extended Data Fig. 4**). In contrast, HA-containing combinations may be preferred for proximal projections. For dense projection regions, single-epitope assignments may facilitate separation of intermingled axonal signals during multiplex decoding, whereas more complex combinatorial

assignments may be better suited for sparse projection labeling. To minimize combinatorial complexity and preserve robust signal detectability, combinations containing fewer epitopes may be preferred.

Extended Data Fig. 6 | Preprocessing pipeline for multiplexed volumetric imaging

a. Flat-field shading correction for illumination normalization. Representative images before and after correction demonstrate removal of spatial illumination inhomogeneity across the imaging field (top). Intensity line profiles (dashed lines) further show improved illumination uniformity following correction (bottom).

b. Chromatic shift correction for cross-channel alignment. Intensity profiles illustrate positional shifts in ch4 before correction (left). Representative overlays of raw ch1 and ch4 images together with corresponding error maps visualize chromatic misalignment prior to registration (middle). Distribution of inter-channel distances before (~6 pixels, 0.62 μm) and after correction demonstrates improved spatial alignment following registration (right). The pre-correction offset falls within the size range of presynaptic boutons (0.5–2 μm).

c. Z-signal correction for depth-dependent attenuation. Raw z-stacks showing progressive signal attenuation across imaging depth are shown on the left, whereas corrected z-stacks demonstrate restored signal uniformity across z-depths on the right.

d. ISCL-based framework for z-signal correction. Histogram matching was first applied to align frame-by-frame intensity distributions to a reference frame across imaging depth. ISCL framework then separated signal from noise while preserving structural features. Fréchet Inception Distance (FID) and Learned Perceptual Image Patch Similarity (LPIPS) metrics demonstrated improved perceptual and structural fidelity following correction.

Extended Data Fig. 7 | Dendrite subtraction and biological rule extraction for synapse assignment

a. Dendrite subtraction for isolation of axonal signals. Adaptive Gaussian mixture model (GMM)-based thresholding and thickness-based morphological segmentation were used to identify and remove dendritic structures from the postsynaptic channel. Remaining axonal signals were subsequently refined using morphological dilation to preserve thin and fragmented structures.

b. Biological rule extraction from synapse-centered binary masks. Overlap rules quantified spatial co-localization using a combined Jaccard–Dice overlap metric (left). Morphology rules extracted orientation, skeleton length, and eccentricity features from axonal structures (middle). Graph rules encoded inter-channel relationships as nodes, edges, and cliques to capture higher-order co-localization patterns and combinatorial channel associations, from which graph-based metrics were derived (right).

Extended Data Fig. 8 | SynScope webUI and interactive analysis workflow

a. Web-based implementation of the SynScope platform. A modular node-based workflow for data input, preprocessing, puncta assignment, and visualization is shown on the left. An example SynScope WebUI together with an interactive puncta visualization interface is shown on the right. A step-by-step demonstration of the complete SynScope workflow is provided in **Supplementary Movie 1**.

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