Li *et al. Journal of NeuroEngineering and Rehabilitation* (2025) 22:118
https://doi.org/10.1186/s12984-025-01655-3

Journal of NeuroEngineering and Rehabilitation

REVIEW Open Access

Reveal the mechanism of brain function with fluorescence microscopy at single-cell resolution: from neural decoding to encoding

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Abstract

As a key pathway for understanding behavior, cognition, and emotion, neural decoding and encoding provide effective tools to bridge the gap between neural mechanisms and imaging recordings, especially at single-cell resolution. While neural decoding aims to establish an interpretable theory of how complex biological behaviors are represented in neural activities, neural encoding focuses on manipulating behaviors through the stimulation of specific neurons. We thoroughly analyze the application of fluorescence imaging techniques, particularly two-photon fluorescence imaging, in decoding neural activities, showcasing the theoretical analysis and technological advancements from imaging recording to behavioral manipulation. For decoding models, we compared linear and nonlinear methods, including independent component analysis, random forests, and support vector machines, highlighting their capabilities to reveal the intricate mapping between neural activity and behavior. By employing synthetic stimuli via optogenetics, fundamental principles of neural encoding are further explored. We elucidate various encoding types based on different stimulus paradigms—quantity encoding, spatial encoding, temporal encoding, and frequency encoding—enhancing our understanding of how the brain represents and processes information. We believe that fluorescence imaging-based neural decoding and encoding techniques have deepened our understanding of the brain, and hold great potential in paving the way for future neuroscience research and clinical applications.

Keywords Neural imaging, Mathematical model, Optogenetics, Neural decoding, Neural encoding

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Introduction

Single-cell resolution imaging offers precise recordings of the morphology and activity of neurons, facilitating the comprehension of the organizational principles underlying brain function and enabling the construction of more authentic neural network models [1]. Compared with traditional imaging techniques, such as EEG and fMRI, which are only capable of recording neural activities at significant population levels, two-photon fluorescence imaging, with its exceptional deep tissue penetration, high spatial resolution, and low phototoxicity, has become an essential tool for exploring brain functions in living animals [2]. This technique allows researchers to observe the structure and function of the brain in living animals in depth without causing significant damage to the samples, achieving long-term, continuous dynamic monitoring. Especially in terms of single-cell resolution, the two-photon fluorescence imaging technique has overcome the limitations of traditional imaging techniques, accurately capturing the fluorescence changes of individual neurons, revealing the subtle processes of intracellular signal transduction and the complex communication between cells, thereby facilitating the analysis of neural mechanisms [3]. As one of the crucial applications of two-photon fluorescence imaging techniques, two-photon calcium imaging (2PCI) uses calcium-sensitive fluorescent indicators, such as genetically encoded calcium indicators (GECIs) like the CaMP series, to record changes in intracellular calcium ion concentration during neural activities. In addition to GECIs, a variety of chemical calcium-sensitive fluorescent indicators, such as Fluo-4 and Fura-2, have also been widely utilized. When neurons fire action potentials, the intracellular calcium level increases, and this change can be accurately recorded, making 2PCI a powerful tool in neuroscience research.

The groundbreaking advancements in the two-photon fluorescence imaging technique have provided new perspectives and tools for studying neural activities, mainly neural decoding methods, due to its high spatial and temporal resolution and its ability to monitor neural activities in vivo [4]. Neural decoding refers to the process of translating behaviors, sensory recognition processes, internal brain states, or cognitive intentions into interpretable neural signal representations [5]. These neural states may include overt behaviors and conscious mental processes, as well as unconscious motor activity under anesthesia, or pathological events such as epileptic seizures and disturbances of consciousness [6–10]. It has many applications, including understanding how sensory systems encode external stimuli, how motor systems generate and execute actions, and how pathological or latent brain states are represented with neural dynamics.

Although this review primarily focuses on fluorescence imaging-based approaches for neural decoding, it is important to position these techniques within the broader context of neurophysiological recording strategies. Electrophysiology-based methods, such as spike sorting, remain widely used due to their millisecond-level temporal resolution [11]. In contrast, imaging techniques, despite having lower temporal fidelity, offer single-cell spatial resolution and the ability to monitor large populations of neurons. These distinctions highlight the complementary strengths of electrophysiology and imaging, and support the rationale for focusing on the unique contributions of imaging-based decoding approaches at single-cell resolution.

While neural decoding reveals how the brain connects external behaviors with internal neural activity representations, the field of neural encoding seeks to integrate information from multiple analytical levels. It explains how the collaborative activities of neurons produce corresponding behaviors. The basis of this cascade of information processing is the information converted and transmitted by individually stimulated neurons, which is manifested through evoked action potentials. Therefore, the sequence of action potentials characterized by specific neurons provides information for the neuron's projection target. Considering the stimulation method, the encoding patterns fall into quantity encoding, spatial encoding, temporal encoding, and frequency encoding. Frequency encoding is the most common form of encoding, where the firing rate of a neuron is proportional to the intensity or characteristics of the stimulation [12]. Different numbers of neurons in corresponding trials are selected for quantity encoding as targets during stimulation. In temporal encoding, the designed stimulation temporal sequence will be applied to a particular neuron group, and the neuron's response to the stimuli could be precisely timed, with the order of spikes encoding different information [13]. On the other hand, spatial encoding uses the spatial map of individual neurons and their responses to specific spatial locations to encode information [14]. Just as decoding serves as the conduit for interpreting incoming information, encoding functions as the mechanism that translates the brain's internal decisions and intentions into tangible actions. Together, these two processes constitute a complete cycle of information processing within the nervous system, thereby ensuring the generation of adaptive behavioral responses, such as those elicited by environmental stimuli.

As a powerful tool for studying the relationship between specific neural circuits and brain functions, [15, 16] optogenetics is commonly used to create synthetic stimuli by replacing natural stimuli, thereby providing well-controlled experimental conditions with precise parameterization and causal manipulation, to facilitate

the exploration of the fundamental principles of neural encoding [17]. Optogenetics combines the advantages of genetics and optics, [18] allowing researchers to precisely control the activity of specific cells in spatial and temporal domains using light. It involves inserting the genes of light-sensitive proteins, such as ChR2[17] and Halorhodopsin, [18] into nerve cells, causing them to express corresponding ion channels that either inhibit or activate neural activity [19]. Usually, the process includes the following four steps: (1) Finding suitable light-sensitive proteins; (2) Introducing the genes of light-sensitive proteins into target cells; (3) Controlling optical signals in spatial and temporal domain; (4) Collecting output signals and reading results [20]. Compared with traditional research methods, optogenetics has distinct advantages [21, 22]: (1) High spatial and temporal precision: using light as a stimulus medium, millisecond-level control of neural activity can be achieved, and specific cells can be regulated through tissue-specific promoters; (2) Controllable stimulus intensity: laser modulation enables precise adjustment of stimulus intensity; (3) Reversibility: light activation is reversible, and cellular activity can be turned off by stopping the light exposure; (4) Minimal invasiveness: no foreign material is introduced into the tissue. Specifically, two-photon holographic optogenetics, as a cutting-edge technique capable of controlling and monitoring neural activity, has been rapidly applied and developed in scientific research for single-cell-resolution manipulation of neural activity in 2D or 3D space, which is crucial for a deeper understanding of the relationship between neural encoding and perception, cognition, and behavior. Currently, two-photon holographic optogenetics falls into the following four categories: spiral scanning computer-generated holography (CGH), [23, 24] scanless 2D-CGH with temporal focusing (TF), [25] scanless 3D-CGH with TF (3D-SHOT), [26] and multiplexed scanless 3D-CGH with TF (MTF-CGH)[27]. In addition to traditional optogenetic stimulation using visible light, recent research has introduced hybrid approaches that integrate radiation (such as X-rays or near-infrared light), nanoparticles, and optogenetic tools. For instance, Chen demonstrated a strategy in which X-ray-excitable nanoparticles were used to remotely activate opsin-expressing neurons, enabling deep brain modulation without requiring invasive light delivery [28]. This hybrid approach represents a promising direction for non-invasive and precise neural control.

In this review, we investigated the mathematical modeling of neural population activities, decoding methods, and encoding methods from the perspective of single-cell resolution and the complete cycle of interpretation (neural decoding) and validation (neural encoding). We categorize the decoding methods into linear, nonlinear, and hybrid models, including independent component analysis (ICA), random forests (RF), support vector machines (SVM), and others. Based on stimulus patterns, we summarize the encoding methods into the following categories: quantity encoding, spatial encoding, temporal encoding, and frequency encoding. It should be noted that we emphasize the basic unit, i.e., the single neuron, in recording and manipulation of neural activity in vivo. Further, we discussed the potential of influencing neural networks based on Hebbian theory with neural decoding and encoding techniques, the limitations of two-photon imaging, and the critical role of neural decoding and encoding in the development of brain-computer interfaces (BCIs).

The mathematical modeling of neural activities

The mathematical modeling process of neural activities is a crucial step in converting complex neural activity data into a mathematical representation (Fig. 1). Initially, neural activity data is captured using techniques such as calcium fluorescence imaging, and behavioral data related to these activities, such as movement trajectories and specific behavioral expressions, is simultaneously recorded. After data collection, preprocessing is necessary, including noise removal and standardization, to ensure the quality and consistency of the data [29]. Next, feature extraction and selection are performed, where neural activity data is typically segmented into multiple time windows, within which features such as spike frequency, inter-spike intervals, and fluorescence intensity are extracted. These features can be further analyzed and refined using statistical methods or signal processing techniques [30]. Another approach to feature extraction and selection is feature vectorization, which involves converting neural activity into vector form to facilitate analysis. This process may incorporate techniques such as normalization, binarization, mapping, and dimensionality reduction, all of which are designed to enhance the model's generalization capabilities and interpretability [5]. Next, the modeling stage employs various mathematical and computational methods to capture the relationship between neural activity and behavior. Linear regression models assume a linear relationship, while nonlinear models such as SVM and logistic regression (LR) can capture more complex relationships. These models are essential for understanding how external information is translated into neural activity and for developing effective decoding strategies. As model evaluation is vital to ensure effectiveness, cross-validation or performance assessment is usually applied to determine the model's predictive capability and potential applications. Ultimately, a successful decoding model can translate new neural activity data into corresponding behavioral data, enabling prediction and analysis [31]. This process is significant not only in neuroscience

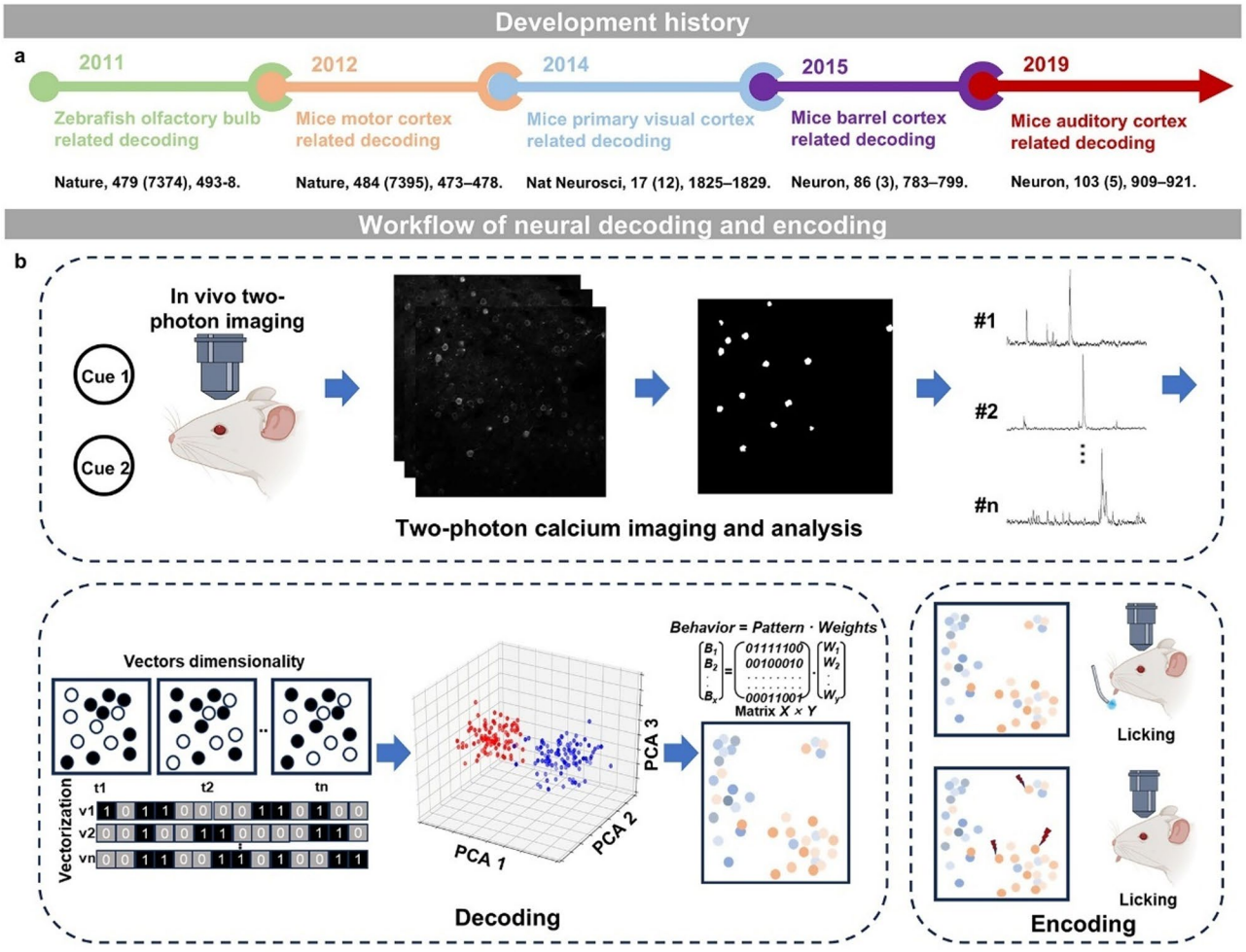

Fig. 1 The history and workflow of neural decoding and encoding. (**a**) The development history of neural decoding and encoding. (**b**) The typical workflow of neural decoding and encoding begins with in vivo two-photon calcium imaging in mice exposed to various cues. The captured real-time neural activity signals are vectorized and subjected to dimensionality reduction to visualize distinct neural activity patterns. The decoding phase involves predicting behavioral patterns from these neural signals, while the encoding phase involves manipulating specific neural activity patterns to control behavior. This workflow highlights the integration of advanced imaging, data analysis, and behavioral modulation in understanding neural coding mechanisms

research for uncovering the relationship between neural activity and behavior and understanding brain encoding mechanisms, but also in practical applications. For example, in the field of BCIs, decoding brain signals to control external devices offers new solutions for medical rehabilitation and human-computer interaction.

Neural decoding method

Linear model

Independent component analysis

Independent component analysis is a linear signal separation technique that enables researchers to extract statistically independent signal sources, known as independent components, from complex neural network activities (Fig. 3a). These components represent the fundamental activity patterns within the network, which are statistically uncorrelated. This characteristic aids researchers in identifying neural activity patterns associated with specific behaviors. To deepen the understanding of the connection between neural ensembles in the nucleus of the medial longitudinal fascicle (nMLF) of the zebrafish brain and the behavior of tail bending, [32] ICA was effectively utilized in the study. By combining ICA with regularized regression models, researchers were able to pinpoint subsets of neurons that significantly influence specific behavioral parameters, such as changes in the tail angle. This approach revealed the specific neural populations within the nMLF area that are instrumental in regulating behaviors and the collaborative mechanisms through which these neurons produce behavioral outputs. Additionally, through the photoactivation of the photosensitive protein paGFP, researchers performed a morphological reconstruction of these functionally significant neurons, providing detailed anatomical structure and connectivity

information. This approach offers a comprehensive perspective on how the brain controls behavior.

In another study, the ICA algorithm was employed to achieve the decoding of specific neural populations with single-cell resolution [33]. Concentrating on the primary visual cortex (V1) and the lateromedial (LM) visual area of mice, they utilized a dual-axis two-photon microscope in tandem with the calcium indicator GCaMP6s to monitor neural activity in the mouse brain. During the experiments, the mice, with heads restrained but free to run, were subjected to visual stimulation via moving grating patterns. The research discovered a supralinear relationship between the number of active neurons in the LM area and those in the V1 area under the same visual stimulation conditions. By applying ICA and principal component analysis (PCA), the calcium transients of individual neurons from the imaging data were extracted, which facilitated the identification of the engaged neural communities.

Principal component analysis

Principal component analysis is a statistical procedure that transforms a set of potentially correlated variables into a set of linearly uncorrelated variables known as principal components (Fig. 3a). In the decoding analysis of neural activity, PCA assists in extracting critical features from high-dimensional neural data, thereby reducing dimensionality and noise. These features encapsulate the predominant patterns of activity within neural ensembles. As previously discussed, PCA is adept at decoding calcium transients of individual neurons [33]. In another study, PCA was utilized to decode neural ensembles associated with visual responses. Researchers employed two-photon imaging and two-photon holographic optogenetics to observe neural activity in the L2/3 of the mouse V1. The mice were conditioned to respond to orthogonal drifting-gratings in a Go/No-Go visual task, licking to receive water rewards upon Go signals and withholding licking to avoid high-frequency noise punishment during No-Go signals (Fig. 2d). By applying PCA, the researchers identified a close relationship between the activation of Go ensemble neurons and the licking behavior of the mice, and a corresponding relationship between the activation of No-Go ensemble neurons and the suppression of licking. This study demonstrates the role of neural ensembles in the mouse V1 in decoding visual information and controlling visually guided behaviors, providing new perspectives on how the brain processes visual information and establishing a foundation for potential clinical applications in the future [34].

Naive bayes classifier

The naive Bayes classifier (NBC) is a simple probabilistic classifier based on Bayes' theorem, which makes predictions by calculating the probability of a category given the input features (Fig. 3a). Utilizing this algorithm, the activity of specific neural populations in the subiculum, i.e., corner cells, has been decoded with single-cell resolution [35]. Researchers first recorded the activity of mice in open arenas of various geometric shapes, including circles, equilateral triangles, squares, and hexagons, using a single-photon miniscope. They discovered that corner cells exhibited heightened neural activity as the mice approached corners, and that these cells modulate the activity in response to the angle of the corners, the height of the walls, and the degree of wall intersection, thus responding to both concave and convex corners in the environment. In this study, the NBC took the binarized, deconvolved spike activity from all recorded subicular neurons as input features, associating these features with the positional labels of the mouse in the environment to train the model. After training, the model could predict the precise location of the mouse in the environment based on new neural activity data, especially near corners. This method demonstrates the encoding capabilities of corner cells for specific geometric features in the environment, and through the application of the NBC, reveals how these cells provide information for spatial navigation and position. Consequently, researchers have been able to directly link neural activity to the navigational behavior of mice in complex environments, offering a new perspective on how the brain constructs a cognitive map of the environment.

Besides, the NBC was utilized to explore the neural mechanisms of heading representation in spatial cognition. Researchers employed 2PCI to record the calcium transients of neural populations in the retrosplenial cortex (RSC) of mice. In the experiments, mice with their heads restrained moved freely within an environment, enriched with visual cues such as solitary black cues or star patterns. By implementing a controlled rotational setup, the study was conducted under both light and dark conditions to examine the influence of visual input on heading perception. The research team applied the NBC method to analyze the calcium signals of the neural populations, revealing three functional types of neurons within the RSC: (1) Heading cells, which consistently decoded heading accurately under both light and dark conditions; (2) Landmark cells, which predominantly relied on visual cues under light conditions; (3) Alignment cells, which adeptly used visual cues under light conditions and sustained decoding accuracy in the dark [36]. These insights are instrumental for comprehending how the brain manages spatial information.

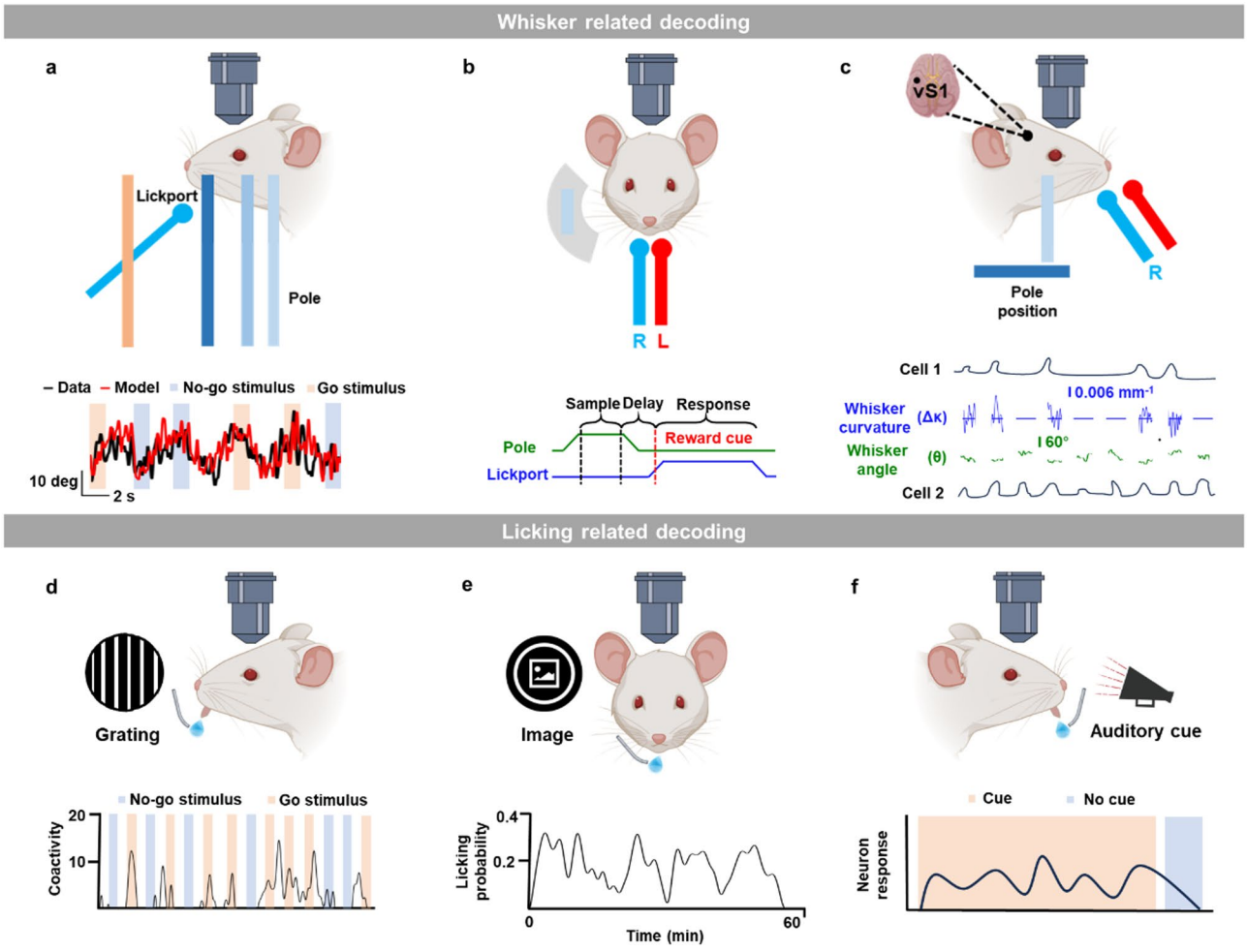

Fig. 2 Whisker- and licking-related decoding. (**a**) Mice were head-fixed under a two-photon microscope and trained on a whisker-based detection task, where correct detection of a pole resulted in a water reward when reported by licking. The waveform compares actual whisker movement with predictions based on neural activity, demonstrating the model's accuracy. (**b**) Mice selected the appropriate lickport based on pole position, with the reward signaled by an auditory cue after a delay period. The diagram shows the sample, delayS, and response epochs. (**c**) Neurons in the vS1 responded to different whisker dynamics—Cell 1 to curvature and Cell 2 to angle changes. (**d**) Licking behavior in response to visual grating stimuli shows increased coactivity during go trials compared to no-go trials. (**e**) Licking probability fluctuated over time when presented with natural image stimuli. (**f**) Auditory cues significantly increased neuronal responses linked to licking behavior, whereas the absence of cues led to reduced activity

Non-linear model (machine learning)

Random forest

Random forest is a machine learning algorithm that enhances accuracy by constructing multiple decision trees to address classification or regression problems (Fig. 3b). Known for its robust predictive power and ability to model nonlinear relationships within datasets, RF has been extensively applied in neuroscience research for decoding and pattern recognition tasks. A pioneering study utilized long-term imaging technology, in conjunction with the genetically encoded calcium indicator GCaMP3, to track the activity of neurons in the L2/3 region of the mouse motor cortex [37]. The researchers developed a behavioral task in which mice detected objects with their whiskers and reported the detection through licking behavior (Fig. 2a). Subsequently, they employed the RF algorithm to decode the complex associations between neural activity and the mice's behavioral performance, revealing that the neural populations related to tactile and motor behaviors (such as whisker movements and licking) were spatially intermixed. As the learning process advanced, the population-level representations became more stable and redundant, despite the dynamic changes in the representations of individual neurons. This suggests that during sensorimotor learning, neural ensembles in the motor cortex are capable of linking sensory inputs with specific motor programs.

To elucidate the link between behavioral strategies and neural activity, researchers employed the RF algorithm to decode the neural activity in V1 and LM of mice during a visual change detection task, with recordings made using 2PCI. In the experiment, mice were tasked

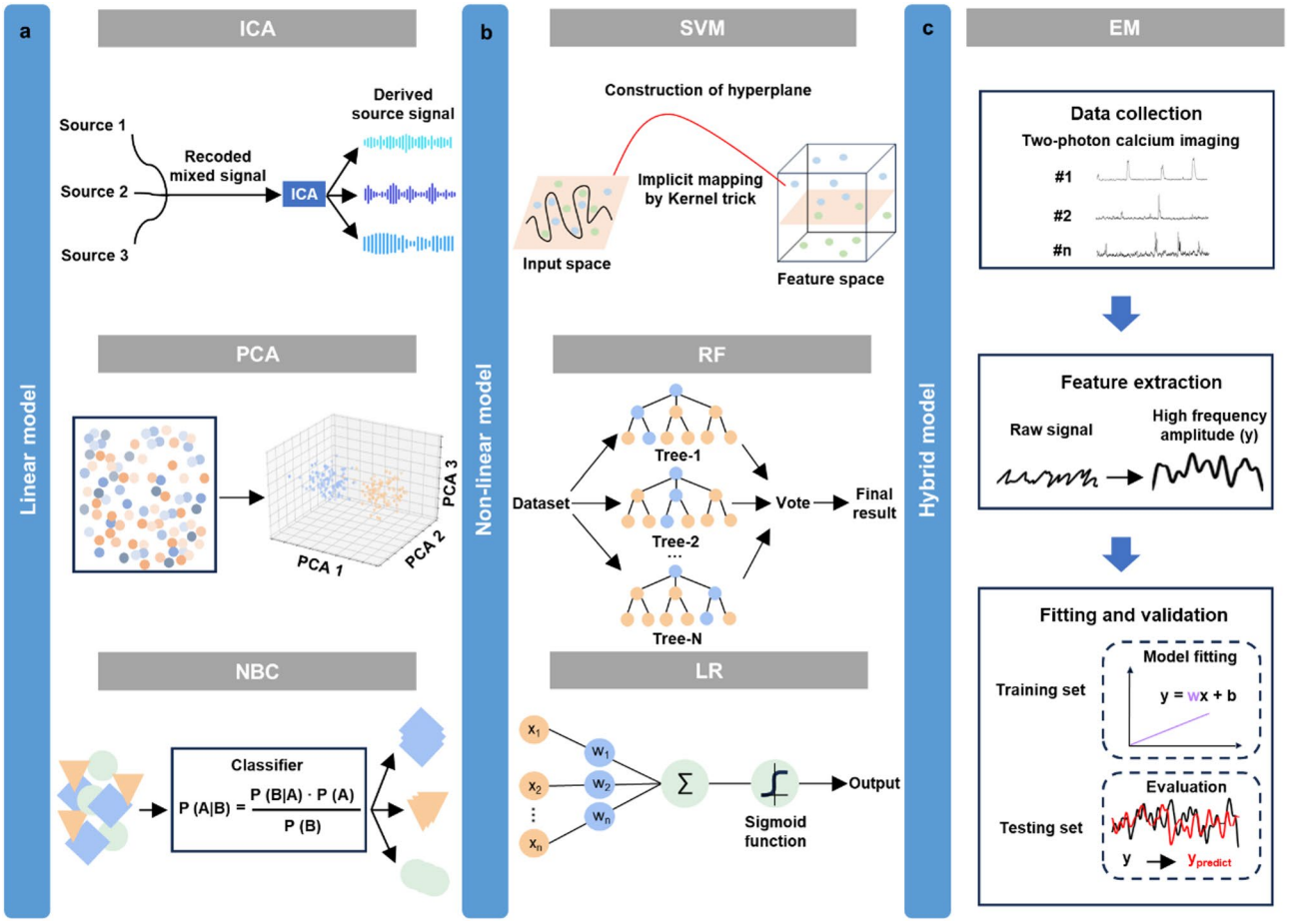

Fig. 3 Schematic diagram of the decoding methods. The specific decoding methods include (**a**) linear models, (**b**) non-linear models, and (**c**) hybrid models. (**a**) Linear models encompass ICA, PCA, and NBC, among others. (**b**) Non-linear models include SVM, RF, and LR, among others. (**c**) Hybrid models include EM, among others

with comparing presented natural images and received rewards for detecting changes through licking behavior (Fig. 2e). The findings revealed that the visual comparison strategy utilized by the mice during task execution significantly influenced the activity of the excitatory neural population. By employing the RF algorithm, the investigators decoded a strong correlation between the activity of this excitatory neural group and the mice's behavioral choices, with an increase in neural activity observed prior to the mice's detection of visual changes and the subsequent licking response [38]. This study aids in uncovering the neural mechanisms of sensory processing that are contingent upon strategic behavior.

In another study, the RF algorithm was employed to decode the specific location of mice within a virtual tunnel and the identity of the grating stimuli they encountered. The research concentrated on neurons in the L2/3 of the mouse's V1. The mice, with their heads secured, were allowed to run freely on a spherical treadmill as they explored a virtual tunnel. Utilizing two-photon imaging with the GCaMP6f calcium indicator, the researchers recorded neural activity in response to various visual stimuli within the tunnel to investigate the mice's predictive reactions to visual cues. Upon training an RF classifier, the study revealed that neural activity in V1 became increasingly informative as the mice gained experience in a specific environment, particularly in anticipating upcoming visual stimuli. The discovery of predictive neurons was highlighted, demonstrating spatially contingent anticipatory activity prior to the presentation of expected visual stimuli [39].

Support vector machine

The support vector machine classifier is a machine learning algorithm capable of accurately predicting whether a mouse is exploring or merely moving based on patterns of neural activity (Fig. 3b), thereby decoding the association between the activity of neurons in the mouse hippocampal CA1 region and behavior. By employing two-photon fluorescence imaging to analyze the activity differences of these neurons during object exploration versus non-exploration periods, researchers identified a unique type

of neuron, known as object exploration-dependent place cells (oePCs). These oePCs exhibit increased activity prior to a mouse's decision to explore an object and are nearly inactive when the mouse passes through the exact location without exploration [40]. Utilizing the SVM classifier, researchers revealed that the activity of oePCs serves as a neural representation of the mouse's exploratory intentions, independent of the mouse's movement trajectory, changes in speed, or visual perception of the object. Furthermore, to explore the regulatory mechanisms of these neural activities, researchers used chemogenetic techniques to inhibit the activity of the lateral entorhinal cortex (LEC), a crucial input region of the hippocampus responsible for conveying non-spatial sensory information about objects and space. The findings indicated that when the LEC is suppressed, the activity of oePCs during exploration significantly decreases, demonstrating the essential role of the LEC in encoding exploratory intentions by hippocampal neurons. This study sheds light on how specific neurons in the mouse hippocampal CA1 region encode the intention to explore and illustrates the direct link between these neural activities and the exploratory behavior of mice, enhancing our understanding of the hippocampus's role in spatial cognition and behavioral control.

Logistic regression

Logistic regression is a statistical method used to analyze the relationship between one or more independent variables, such as neural activity, and a binary dependent variable, such as a specific behavioral outcome (Fig. 3b). It makes predictions by estimating the influence of the independent variables on the probability of an event occurring. In particular, this model was utilized to assess the relationship between the activity of specific neural populations in the brain of zebrafish larvae and their behavioral performance [32]. Specifically, researchers first employed two-photon holographic optogenetics and volumetric imaging to precisely activate and record neural populations in the larval zebrafish brain. Subsequently, critical patterns in network activity were extracted using ICA. Building on this foundation, LR models were applied to quantify the contribution of each neuron to the specific behavioral outcome of tail bending. This model helped differentiate neural activities associated with tail bending and swimming behaviors, and also revealed how these neurons, located in the nMLF, collectively regulate behavioral performance. Through these methods, researchers successfully decoded the neural activity related to the tail movement behavior of zebrafish larvae, providing new perspectives and tools for understanding how neural circuits control behavior.

Conditional random field

The conditional random field (CRF) is a probabilistic model frequently applied to sequence labeling and modeling tasks. Given that neural activity often manifests as time-series data, CRFs have found extensive use in neural decoding. To simulate and comprehend the activity patterns within neural ensembles, CRFs have been employed to analyze data recorded from neural ensembles in the V1 of mice. Researchers utilize CRF models to quantify the contribution of each neuron to the collective activity, and identify neurons with pattern completion capabilities. It is revealed that even when only a subset of neurons is activated, there is a potential to initiate activity across the entire ensemble [34].

Hybrid model

Using two-photon imaging combined with the genetically encoded calcium indicator GCaMP6s, researchers conducted an in-depth analysis of the calcium dynamics of L2/3 pyramidal neurons in mice. To decode the specific links between these neural activities and tactile behaviors, a statistical method known as the encoding model (EM) was employed [41] (Fig. 2b). This model correlates behavioral variables, such as the angle and curvature changes of whiskers, with neural activity, quantifying how individual neurons respond to specific tactile stimuli. The EM comprises two principal components (Fig. 3c). First, it utilizes static, point-wise nonlinearities to process input variables, such as whisker motion, capturing the nonlinear response characteristics of neurons to tactile stimuli. Second, the model employs a linear temporal kernel to simulate the temporal dynamics of neural responses, accounting for the delay and duration from stimulus occurrence to neural activity changes. Through this model, researchers can assess the encoding capacity of each neuron for specific behavioral variables and identify which neurons are most sensitive to the direction and intensity of tactile stimuli. Additionally, the study explored the directional selectivity of neurons, that is, their preference for whisker protrusion or retraction movements, further elucidating the complex relationship between neural activity and the tactile decision-making behavior of mice.

In another study, researchers trained mice to perform an object localization task and utilized the EM to predict neural activity based on the kinematics of whisker motion, such as whisker angle (θ) and whisker curvature (κ) [42] (Fig. 2c). This model analyzed the neural responses to whisker movement and tactile stimuli, calculating an encoding score that quantified the correlation between neural activity and tactile input. To further explore the direct link between specific neural populations and behavioral performance, the researchers employed a multiphoton ablation technique to selectively

inactivate neurons that stood out in terms of encoding scores. By comparing neural activity and task performance before and after ablation, the study revealed the role of touch neurons and whisking neurons in somatosensory cortex L2/3, in mice performing tactile discrimination tasks, also achieving single-cell resolution in decoding. The application of the EM enabled researchers to accurately identify and decode the neural populations involved in tactile processing, establishing a direct link between neural activity and the mice's tactile exploration and object localization behaviors.

These decoding strategies vary in their performance depending on algorithmic complexity, input features, and behavioral context, and have been extensively applied in small animal models such as rodents and zebrafish. Importantly, similar approaches have also been extended to nonhuman primates. For instance, optical decoding of arm movement trajectories has been achieved in rhesus macaques using dendritic calcium signals recorded through two-photon imaging, enabling real-time control of an optical brain-computer interface [43]. In addition, high-dimensional decoding of sequential working memory in the prefrontal cortex has revealed a geometrically disentangled representation of spatial and ordinal information, providing a mechanistic basis for sequential behavior [44]. These findings underscore the translational potential of neural decoding frameworks and their applicability across species and cognitive domains.

To facilitate method selection in experimental and computational studies, we summarize the key characteristics of commonly used neural decoding algorithms in Table S1.

Neural encoding method

Quantity stimulation pattern

The concept of neural quantity encoding typically refers to the method in which activation depends on the number of neurons within an ensemble (Fig. 5a). In this encoding method, the stimulus is represented by activating a certain number of neurons, rather than relying on the firing frequency or timing of individual neurons. This type of encoding emphasizes the coordinated effect of the neural population and how different neurons work together to respond to external stimuli.

In a study examining the cellular groups involved in mouse perceptual responses, synthetic optogenetic odors were used to control the olfactory perception of mice and measure behavioral reactions, revealing the logic of perceptual encoding. By varying the number of stimulated neurons, the experiment demonstrated that the accuracy of stimulus detection in mice declined as the number of activated neurons decreased, highlighting the importance of neural population size in perceptual encoding [45]. In another study focused on cellular groups involved in mouse visual responses, a technique combining two-photon optogenetics and two-photon calcium imaging was employed to measure and manipulate neural activity in the mouse visual cortex, exploring the role of neurons in visual stimulus processing. The results showed that when more than 80 neurons were stimulated, mice performed better in behavioral tasks, indicating that the coordinated activity of neural populations plays a critical role in behavioral output [23].

In a study of behavioral decision-making during tactile discrimination tasks in mice, two-photon calcium imaging combined with two-photon optogenetics was employed to reveal key neural dynamics involved in the decision-making process. The experiment stimulated different numbers of neurons in the second and third layers of the primary sensory cortex (S1) to examine their effects on mouse behavior. The results indicated that decision neurons exhibited no clear decision-related signals in error trials, thereby confirming the importance of decision signals in the decision-making process [46].

Spatial stimulation pattern

Neural spatial encoding refers to the concept that the spatial distribution of activated neurons influences the behavioral outcomes (Fig. 5b). In spatial encoding theory, neurons with different locations play different roles in representing and processing information. The arrangement of neurons in the cerebral cortex is a form of spatial encoding, implying that the physical layout of neurons in the brain and their interactions are key factors in encoding information.

To investigate the role of hippocampal neurons in the exploratory behavior of mice, an experiment was conducted in which mice were trained to move along a specific trajectory in an environment with fixed cues, allowing them to freely pause and explore objects they had previously encountered. The experiment involved optogenetic inhibition of the LEC in mice to observe its effects on hippocampal neuron activity. The results showed that oePCs became active before the exploration behavior at specific locations and LEC inhibition reduced the activity of these cells during the exploration period [40]. Furthermore, another study employed a combination of two-photon imaging and two-photon optogenetic stimulation to selectively activate task-related neural ensembles in behaving mice, while simultaneously recording activity from surrounding neurons. By directly manipulating decision-related signals in different regions of layers 2 and 3 of the primary somatosensory cortex (S1), the study demonstrated a clear causal relationship between these signals and behavioral outcomes during decision-making [46].

In an experiment investigating the distinct roles of various brain regions in memory storage, mice were trained

over several weeks on a virtual reality task guided by memory. Throughout the process, optogenetic stimulation or inhibition was used to investigate the role of specific brain regions' neurons in memory formation and consolidation, and cellular-resolution imaging was performed simultaneously on the hippocampus, thalamus, and cortex. The findings revealed that spatially dispersed neural populations exhibit different activity patterns in encoding memories. The hippocampus was found to encode various memories equally, while the anteromedial thalamus preferentially encoded salient memories and gradually increased its correlation with the cortex, promoting overall adjustment and synchronization of the cortex [47]. In another study examining the modulation of licking behavior by neurons in the ventrolateral striatum (VLS) of rats, optogenetic inhibition of both direct and indirect pathway neurons revealed that medium spiny neurons expressing D1-type and D2-type dopamine receptors exert opposite effects on licking frequency and display distinct activity patterns within licking sequences (Fig. 4d). These findings suggest that neural populations in different subregions of the VLS play distinct and specialized roles in regulating licking behavior [48].

By employing optogenetics to activate specific neurons while recording with fluorescence imaging, we can observe the activation of targeted neurons and pinpoint their locations. In a study exploring the neurons related to tail bending in zebrafish (Fig. 4a), the combination of optogenetic activation and functional cell population imaging in brain regions, along with the correlation of functional data and behavioral recordings, was used to identify the ensemble of neurons that initiate basic motor programs for tail bending movements [32]. Similarly, in experiments investigating the neurons involved in horizontal eye movements in zebrafish (Fig. 4c), optogenetic stimulation of brainstem neurons was combined with

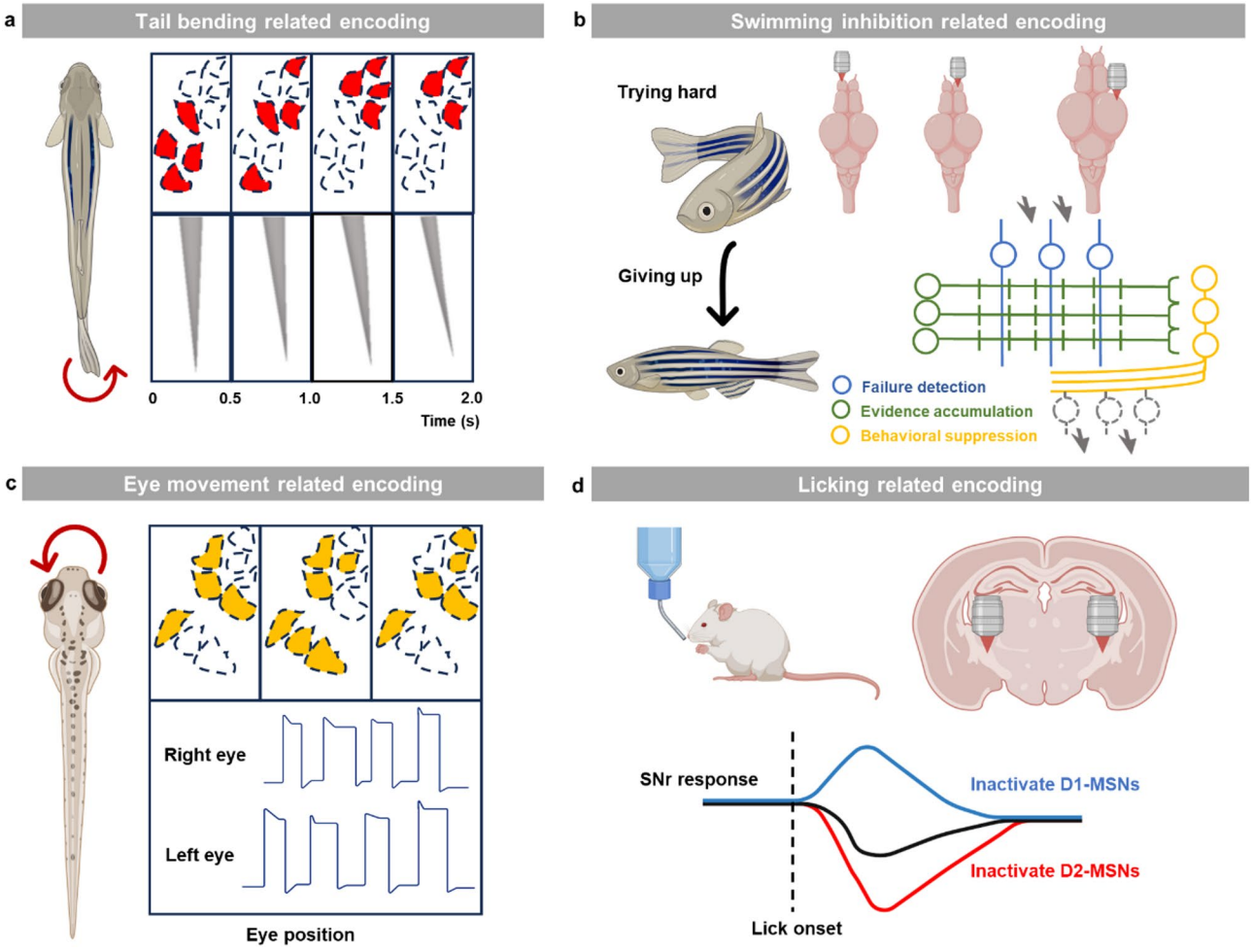

Fig. 4 Neural encoding-related behavioral patterns. (**a**) Optogenetic stimulation of neurons at different locations and their combinations in zebrafish revealed varying tail curvature angles. (**b**) Optogenetic stimulation of neurons at different locations within the pathway during the active-to-passive swimming transition in zebrafish, investigating the roles of these neurons in passive swimming. (**c**) Optogenetic stimulation of neurons at different locations and their combinations in zebrafish showed varying horizontal eye movement angles between left and right eyes. (**d**) Optogenetic inhibition of specific neurons in different pathways revealed opposing effects of inactive D1-MSNs and inactive D2-MSNs in mouse licking behavior

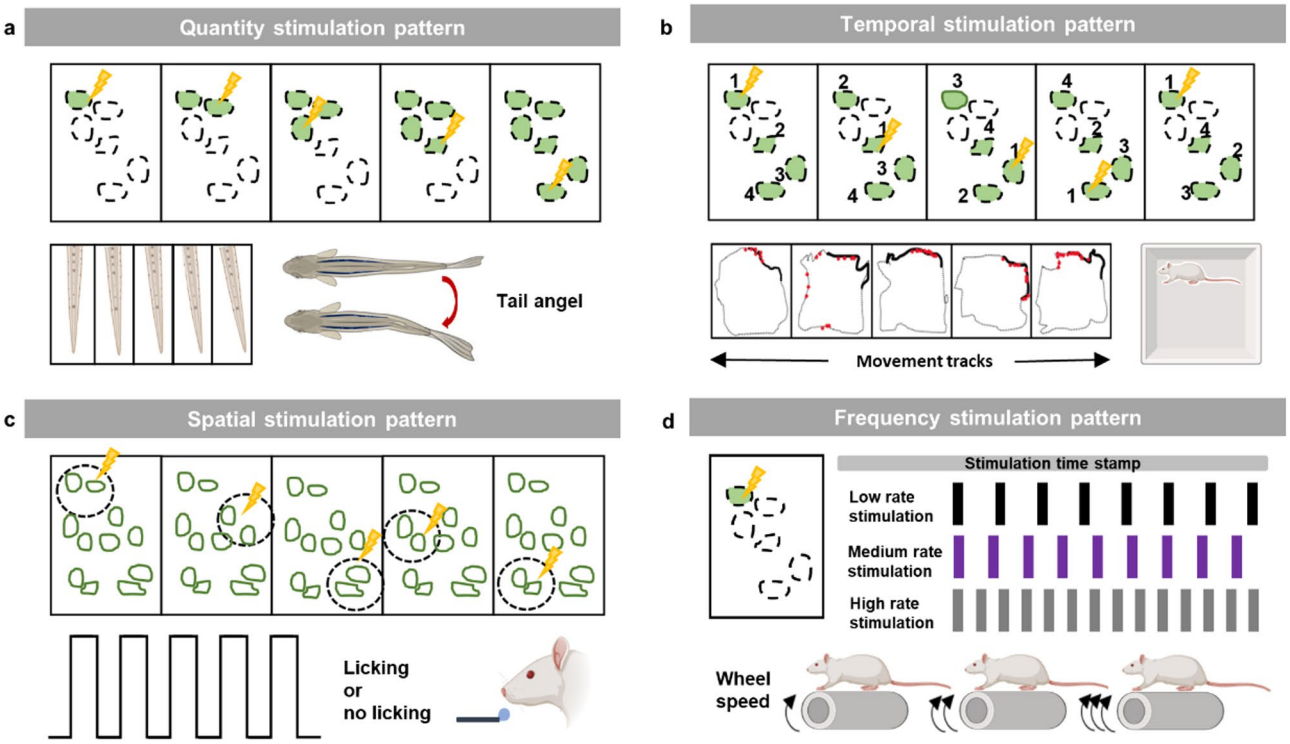

Fig. 5 Classification of encoding stimulation patterns. (**a**) Quantity stimulation pattern: the number of neuronal populations affects the way action potentials are fired, thereby influencing biological behavior, cognition, and sensation. (**b**) Spatial stimulation pattern: usually involves the spatial distribution of neural populations, where the activation patterns across these neurons collectively form a response representation. (**c**) Temporal stimulation pattern: information is encoded through the timing intervals or sequence of firing neurons. (**d**) Frequency stimulation pattern: refers to the method that neurons encode information by changing the frequency of action potentials. In this scheme, the firing rate of a neuron (i.e., the number of action potentials emitted per unit of time) is proportional or inversely proportional to a specific stimulation

two-photon laser scanning imaging during spontaneous eye movements in zebrafish larvae. This approach enabled the precise identification and localization of the neural populations responsible for controlling horizontal eye movements [49]. In a study on passive swimming behavior in zebrafish, optogenetic stimulation of neurons at different locations and imaging of relevant neurons were used to observe the roles of different neurons in altering the fish's behavioral state (Fig. 4b). The results revealed that activation of astrocytes inhibited swimming behavior [50].

Temporal stimulation pattern

Temporal encoding refers to the stimulation delivered to neurons with certain time durations or orders (Fig. 5c). It includes temporal patterns with different stimulation durations, stimulation sequences, and combined temporal stimulation dynamics.

With optogenetic techniques, mice can be trained to perform patterns of activity associated with the light-driven olfactory system, which are referred to as "synthetic odors" [45]. These synthetic odors are generated by activating specific neuron populations with precise spatial and temporal patterns. The perception of these odors in mice depends not only on which neural ensembles are activated, but also on the timing of their activation—analogous to the rhythm and sequence of musical notes. The spatiotemporal template-matching (STM) model further illustrates this principle by assigning weights to the relative timing of neuronal activation within each sequence, highlighting the greater influence of neurons that are activated earlier. In the template, the contribution of individual units is cumulative, with earlier activated units contributing more. Researchers trained mice to recognize synthetic odors constructed by optogenetically induced activation patterns and measured changes in perception under extensive and controllable perturbations in both spatial and temporal dimensions. In subsequent studies, it was further revealed that mice can detect a single spike synchronously generated in fewer than 20 olfactory bulb neurons [51]. Detection performance is strongly dependent on the synchrony of neural activation, rather than the latency relative to inhalation. This indicates that for the behavioral cognition of mice, the synchronous activation of neural populations is a key factor, rather than the relative timing of individual neurons. In another case, [34] changes in mouse behavior were shown to depend on the temporal pattern of neuronal

activation. The timing of stimulation significantly influenced behavioral responses; for instance, the sequential activation of different neural populations was capable of simulating complex perceptual processes. Such precise temporal dynamics are also evident in cognitive functions—stimulated neurons exhibit activity that precedes the peak activation of decision-making neurons, suggesting that sensory inputs are processed prior to the initiation of decision-related neural activity [46]. In the field of memory-related research, by analyzing the temporal patterns of neuronal firing, studies have found the relationship between the precise timing of neuronal firing and memory encoding (including initial short-term memory, memory consolidation, and long-term memory) [47].

Behavior is the fundamental way in which individuals interact with the environment and is crucial for survival, adaptation, and evolution. The activation of neurons plays a decisive role in behavioral cognition and is a key driver of the diversity and complexity of behavior. In licking behavior, D1-expressing medium spiny neurons (D1-MSNs) and D2-expressing medium spiny neurons (D2-MSNs) play roles at different stages of licking [48]. In addition, some studies have shown that astrocytic responses to repeated failed swimming attempts accumulate over time, reflecting the temporal dynamics of glial activity [50]. In research on zebrafish, [32] changes in neural activity after stimulation were observed through repeated optogenetic stimulation strategies, which involve dynamic responses over time. In behavioral analysis, the changes in tail movement after stimulation are associated with the temporal patterns of neural activity.

Frequency stimulation pattern

Frequency encoding is performed by stimulating neurons with different frequencies (Fig. 5d). In this case, the firing frequency of target neurons is expected to be in accordance with the stimulation pattern.

The frequency of neural activity plays a vital role in behavioral decision-making, and studies have found that the behavioral responses of mice are closely linked to the activation frequency of neural populations [34]. Emerging studies suggest that the frequency of neural activity is crucial for modulating behavioral cognition. Experimental modulation of optogenetic stimulation frequency has revealed that mice exhibit frequency-dependent behavioral responses, underscoring the significance of neural activity frequency in cognitive behavior [23]. Additionally, by changing the frequency of optogenetic stimulation, the impact of different stimulus frequencies on mouse behavior was explored, finding that specific frequencies can effectively activate the target neural population and significantly affect mouse behavior [46].

Different frequency stimulation paradigms play an important role in behavior. The latest research [40] found that during exploratory behaviors such as locomotion, olfactory sampling, visual scanning, and object manipulation, the activity frequency of place cells in mice significantly increases. The neural activity in the motor cortex is related to behaviors such as licking, whisker exploration, and whisker touch, and the firing frequency of neurons is proportional to the intensity of the behavior. Researchers trained mice in memory-guided tasks and observed changes in behavioral parameters (such as speed, licking rate), as well as the relationship between neural firing frequency and memory behavior [47]. Similarly, in zebrafish behavioral studies, neuronal firing frequencies associated with swimming were observed to vary across different locomotor states, including initiation attempts, aborted swim bouts, and full cessation [50].

To more clearly distinguish different neural encoding stimulation patterns, in Table S2 we summarize the characteristics, mechanisms, and examples of four different patterns: quantity, spatial, temporal, and frequency.

Discussion and outlook

This review investigates recent breakthroughs in single-cell resolution neural decoding and encoding, highlighting advances achieved through two-photon fluorescence imaging techniques. It covers the entire process from capturing neural activity, preprocessing, feature extraction, modeling, decoding, encoding and validation. By conducting an in-depth analysis, we demonstrate how complex neural signals represent biological behaviors and discuss the potential applications of these techniques. We emphasize the critical role of mathematical modeling in characterizing neural activity dynamics, which provides the fundamental framework for elucidating neural coding principles and enables effective decoding strategies. We systematically evaluate various mathematical and computational methods, including linear models such as ICA and PCA, and nonlinear models such as RF, SVM, and LR. These methods play significant roles in capturing the complex relationships between neural activity and behavior. Notably, we highlight hybrid models, such as EM, which provide new perspectives on understanding neural responses to specific stimuli by combining nonlinear processing and temporal dynamics simulation. These models deepen our understanding of neural activity patterns and offer powerful tools for revealing how the brain processes sensory inputs and controls behavior. Despite significant advances, several challenges remain unresolved. Key directions for future investigation include enhancing decoding accuracy and real-time performance, elucidating interactions between neural populations, and expanding applications to broader biomedical fields. We look forward to future studies overcoming these challenges and advancing neural decoding techniques to deeper and broader applications. In addition, we elaborate on

different types of neural encoding, including quantity encoding, spatial encoding, temporal encoding, and frequency encoding. These encoding schemes are not necessarily mutually exclusive: both individual neurons and neural populations may simultaneously employ multiple encoding strategies to process and transmit information. Understanding the mechanisms of neural encoding helps us comprehend how the complex neural networks in the brain are constructed and coordinated. This understanding aids in diagnosing and treating neurological diseases. As research progresses, new neural mechanisms may be discovered, promoting multimodal data integration and advancing large-scale neural network modeling. Future research may address issues such as overcoming the limitations of biophysical models, performing dynamic decoding analysis, and interpreting artificial neural networks.

Building on the understanding of the complex interactions between neural coding principles and behaviors, we can further explore the significance of these findings for the field of neuroscience and how they influence our understanding of brain function. The Hebbian theory, proposed by Canadian psychologist Donald Hebb, is a classical theory in neuroscience. Its core idea is "neural connection strength is enhanced based on their activity patterns". This "use it or lose it" mechanism, encapsulated in "cells that fire together, wire together", provides a crucial theoretical foundation for understanding learning, memory, and brain function development [52]. In fluorescence-imaging studies of neural coding, Hebbian theory establishes the conceptual framework for analyzing activity-dependent synaptic plasticity underlying information integration. Advanced imaging techniques, particularly two-photon microscopy, enable real-time visualization of both neural ensemble dynamics and synaptic modifications, thereby offering direct experimental validation of Hebbian plasticity principles [53]. Through fluorescence imaging, researchers can observe how specific neural populations enhance or weaken synaptic connections by changing their activity patterns throughout learning and memory formation. For example, in the visual cortex, the synaptic connections between neurons significantly strengthen in animals trained with visual stimuli, vividly illustrating the synaptic weight adjustment predicted by Hebbian theory [38]. Furthermore, fluorescence imaging techniques have revealed how neural populations adjust their encoding strategies through dynamic synaptic plasticity to adapt to constantly changing environmental information. This dynamic encoding mechanism is closely linked to the concept of synaptic weight adjustment in Hebbian theory, exemplifying the computational flexibility and adaptive capacity of neural circuits [54]. Hebbian theory establishes a conceptual framework for understanding neural information processing through synaptic plasticity mechanisms, while fluorescence imaging techniques provide direct visualization of these dynamic processes, thereby bridging the explanatory gap between cellular-level activities and system-level behaviors.

Though 2PCI provides robust support for the experiments of neural decoding and encoding, it is constrained by several methodological challenges and technical limitations. Firstly, in terms of imaging depth, 2PCI relies on the two-photon excitation of near-infrared or infrared light, which scatters as it propagates through brain tissue. The scattering effect confines imaging penetration to superficial cortical layers, compromising visualization of subcortical structures. Additionally, standard 2PCI scans are restricted to a small field of view, meaning that only a small area of a brain region can be imaged at a time. This limitation constrains the imaging field to relatively small cortical areas, preventing the observation of interactions between multiple brain regions. Furthermore, the quality of imaging is affected by several factors. The current imaging techniques face limitations such as uneven expression and distribution of calcium indicators and the presence of photon shot noise, which together result in suboptimal imaging quality. However, recent technological innovations, including dual-axis two-photon microscopy, [33] the continuous development of genetically encoded calcium indicators, [55] and advancements in adaptive optics, [56] have significantly advanced functional brain imaging. These technological developments are progressively enabling more comprehensive and extensive mapping of neural activity patterns.

Although two-photon imaging has some limitations, scientists have widely applied its advantages in scientific researches. In particular, there is a close and fruitful relationship between two-photon imaging and BCI technique. Two-photon microscopy, along with optogenetics, provides important imaging and manipulation tools for the development of BCIs. First, two-photon microscopy enables high-resolution three-dimensional imaging via Z-axis scanning, providing researchers with volumetric visualization capabilities to investigate the brain structure and function in 3D. In the field of BCIs, this provides a critical perspective for understanding how the brain processes information and controls external devices. Second, the combination of two-photon imaging with optogenetics offers a new means of neural manipulation for BCI technology. This technology has been demonstrated in relevant research, [57] where researchers proposed an innovative method of dynamic, patterned, and precise optogenetic stimulation of the mouse cortex in ultra-high-field MRI, heralding the birth of a completely new type of BCI. Additionally, two-photon imaging plays a significant role in the acquisition of neural signals. Researchers have developed a hippocampal BCI that

tests whether rats can voluntarily activate distant place representations by controlling their hippocampal activity [58]. This is another application of two-photon technique in BCIs. In summary, two-photon imaging provides multiple critical functions in BCIs: it enables both structural visualization of deep brain regions and functional characterization of neural circuits, while also facilitating more precise BCI control through integration with optogenetic manipulation and high-fidelity neural signal acquisition. With the ongoing development and refinement of these techniques, future BCI systems are expected to achieve more accurate interpretation of neural intentions for restoring or enhancing brain functions.

Advances in neural decoding not only enhance neuroscience and BCI development, but also contribute to advancing artificial intelligence [59–62]. For instance, stable-state decoding models have enabled accurate reconstruction of complex motor behaviors such as handwriting, offering insights into sequential motor control for intelligent systems [63]. Meanwhile, biologically plausible spiking neurons with reconFigurable firing patterns provide a foundation for neuromorphic computing with improved energy efficiency and enhanced biological realism [64]. These developments highlight the potential of neuroscience-inspired decoding strategies for guiding the design of next-generation intelligent architectures.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12984-025-01655-3.

Supplementary Material 1

Acknowledgements
All Figures were created with http://www.BioRender.com.

Author contributions
Z. Z.: Investigation, Methodology, Writing - review & editing, Funding acquisition, Supervision; K. L.: Investigation, Conceptualization, Methodology, Writing - original draft; H. L.: Investigation, Methodology, Writing - original draft, Visualization; J. Q.: Investigation, Writing - original draft; X. Z.: Investigation, Writing - original draft; B. C.: Writing - review & editing; D. W.: Writing - review & editing, D. Z.: Writing - review & editing, B. L.: Writing - review & editing, H. H.: Writing - review & editing, G. Y.: Writing - review & editing, Supervision.

Funding
The work is supported in part by the National Natural Science Foundation of China (82201637), Zhejiang Provincial Medical and Health Technology Project (2024KY246, 2025KY180), Open Research Fund of the State Key Laboratory of Brain-Machine Intelligence, Zhejiang University (BMI2400025), Key R&D Program of Zhejiang Province (2024C03150), Qiushi Youth Program from Scientific Research Cultivation Foundation (J-202402), Hangzhou Science and Technology Bureau to Z.Z, Zhejiang Health Information Association Research Program (2024XHSZ-Y08) to B.C., National Natural Science Foundation of China (82401786) to H.H.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have approved this manuscript for publication. This manuscript has not previously been published and is not pending publication elsewhere.

Competing interests
The authors declare no competing interests.

Received: 14 March 2025 / Accepted: 17 May 2025
Published online: 27 May 2025

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