0000000040 - OPTIFLEX

SCHADOC: Research and training doctoral programme

First Call Doctoral Fellowships

Optical control of neural circuits for action selection in mice

Acronym: OptiFlex

Primary Supervisor: Dr. Fanny Cazettes
Institut de Neurosciences de la Timone (INT), CNRS, Aix-Marseille University

Co-Supervisor: Dr. Tommaso Fellin
Istituto Italiano di Tecnologia (IIT), Genova, Italy

Non-Academic Partner: Bruker

Thematic Axis: Health and well-being

0000000040 - OPTIFLEX

1. EXCELLENCE (4 pages max)

1.1. Pre-proposal's context, positioning and objective(s)

Scientific context and state-of-the-art: Understanding how the brain flexibly selects and switches between behavioral strategies is a fundamental challenge in systems neuroscience. During natural behaviors like foraging, animals must continuously decide between exploiting current resources (e.g., staying and consuming) and exploring alternatives (e.g., leaving to search elsewhere) (Kolling et al., 2012; Marques et al., 2020). These decisions require the brain to maintain and select among multiple computational strategies, yet the neural circuit mechanisms underlying this flexibility remain poorly understood.

Recent work from the host laboratory has revealed that the mouse secondary motor cortex (M2) simultaneously encodes multiple decision-related signals that could support different foraging strategies, forming a 'reservoir' of parallel computations (Cazettes et al., 2025, 2023). While these signals resemble decision variables at the computational level, it remains unclear how these parallel representations are prioritized at the level of action selection. How does the brain choose between competing motor actions, such as exploiting a known resource or exploring alternatives, and how are transitions between these actions implemented at the neural circuit level? These questions cannot be addressed by recording alone and require causal manipulation of identified neural ensembles with cellular resolution.

All-optical interrogation techniques, combining two-photon (2P) calcium imaging with 2P holographic optogenetic stimulation, now offer unprecedented opportunities to address these questions (Carrillo-Reid et al., 2019; Marshel et al., 2019a; Robinson et al., 2020). These methods allow simultaneous monitoring and manipulation of dozens of identified neurons with single-cell resolution in behaving animals. However, applying these cutting-edge techniques to study cognitive flexibility and action selection remains technically challenging and requires specialized expertise that this project will develop through an international collaboration.

Recent studies of large neural cortical populations suggest that flexible behavior arises from organized patterns of brain activity. Different actions or behavioral states, like exploiting a resource versus exploring for new ones, correspond to distinct patterns of coordinated neural activity (Inagaki et al., 2019; Recanatesi et al., 2022). Switching between behaviors involves transitioning from one activity pattern to another, triggered either by changes in the environment or by spontaneous fluctuations within the brain itself (Tafazoli et al., 2025). Together, these perspectives motivate **the hypothesis that exploit and explore behaviors during foraging correspond to distinct population-level states in secondary motor cortex, and that transitions between them can be causally probed by targeted perturbation of identified neural ensembles**.

Project objectives: This PhD project aims to **reveal the neural ensemble dynamics underlying flexible action selection during foraging**. We propose an innovative, stepwise approach that starts with a tractable question: how does the brain switch between two well-defined motor actions (licking versus running); and then addresses the more general problem of how neural population dynamics stabilize ongoing actions and control transitions between them. This approach ensures feasibility within a 3-year PhD timeline while maintaining the potential for high-impact discoveries.

Specific Objectives:

- Objective 1: Identify and characterize neural ensembles associated with exploitation versus exploration actions. Using longitudinal 2P calcium imaging, identify neuronal ensembles in M2 specifically associated with exploitation (licking) versus exploration (running) behaviors. Test whether these motor actions are encoded in distinct or shared neural subspaces.

- Objective 2: Determine the neural mechanism underlying action transitions. Test two competing hypotheses: (A) specific "switch ensembles" are associated with transitions between exploitation and exploration, or (B) transitions reflect continuous transformation of neural activity without discrete intermediate states.

- Objective 3: Causally test whether targeted 2P photostimulation of identified ensembles can control action selection. Using holographic 2P photostimulation, test whether activating exploitation, exploration,

and switch (in case analysis in Objective 2 support hypothesis (A)) ensembles can bias action selection or trigger behavioral transitions, revealing the functional unit for action switching.

Research hypotheses: We hypothesize that distinct neuronal ensembles in M2 encode exploit versus explore actions in separable subspaces, and that targeted activation of specific ensembles can causally bias action selection. We further hypothesize that transitions between actions are controlled by 'switch ensembles' whose activation triggers state changes in the motor network.

Innovation and originality: This project bridges systems neuroscience and optical engineering by combining: (1) a naturalistic foraging task that requires spontaneous switching between exploitation (licking) and exploration (running) actions, (2) cutting-edge all-optical methods enabling simultaneous recording and manipulation of specific neuronal ensembles, and (3) computational approaches to identify neural activity patterns controlling action transitions. The project will establish causal links between M2 ensemble dynamics and action selection in a complex and self-paced behavioral paradigm, advancing beyond simpler sensory tasks where all-optical methods have been primarily applied (Carrillo-Reid et al., 2016; Gill et al., 2020; Marshel et al., 2019b; Shin et al., 2025).

Methodology - Behavioral paradigm: The project will use a foraging task previously developed by the host laboratory, in which head-fixed mice **forage in virtual reality** (Fig. 1A). Animals alternate between sustained licking at a reward port and running to leave the current foraging site. During each visit of a foraging site, rewards are delivered probabilistically, and once a site becomes depleted, it remains unrewarding, prompting the animal to leave and search for another site. As a result, animals repeatedly and spontaneously switch between two well-defined motor actions: licking (exploit) at a reward site and running (explore) on a treadmill. Importantly, this task admits multiple decision strategies and decision-related signals, which previous work has shown are simultaneously represented in M2. In the present project, this richness is leveraged to study how neural population dynamics resolve competing representations into concrete action selection.

Methodology - All-optical configuration: The setup leverages the host laboratory's newly acquired all-interrogation system consisting of two powerful lasers (CARBIDE CB3 40W and Coherent Discovery) combined with a Spatial Light Modulator (SLM) integrated into an Ultima 2Pplus microscope (Bruker). This dual-laser configuration allows to both image and stimulate neurons simultaneously. The **imaging path** uses the Coherent Discovery laser to excite the green calcium indicator GCaMP8m at ~920 nm to monitor neural activity. The **stimulation path** uses the CARBIDE laser to excite a red-shifted opsin (e.g., Chrimson R (Klapoetke et al., 2014) or rsChRmine (Kishi et al., 2022) but see Part 3. Implementation for more details) at 1030 nm.

Methodology - Experimental protocol: The experiment will follow a stepwise "read-write" logic to test the causal role of M2 ensembles in maintaining actions and switching between them. **(1) Ensemble identification (Read):** We will first record neural activity in M2 during the foraging task to identify specific neuronal ensembles (Fig. 1B). Using dimensionality reduction and clustering analysis, we will classify neurons that are preferentially active during exploitation (sustained licking) versus exploration (running) (*Objective 1*), as well as putative "switch ensembles" that emerge specifically during the transition between these states (*Objective 2*) (Fig. 1D). **(2) Holographic pattern generation:** Based on these recordings, we will compute phase masks for the SLM to target specific subsets of neurons (from 2 to dozens). We will preferentially target neurons that show high selectivity for a single action type. **(3) Targeted 2P stimulation (Write):** We will "replay" the identified spatiotemporal patterns to bias behavior (*Objective 3*) (Fig. 1C). To test if actions can be reinforced, we will stimulate "exploitation" ensembles during potential leaving moments to test if we can prolong the staying/licking behavior. Conversely, we will stimulate "exploration" ensembles to bias the animal toward leaving (Fig. 1E, left). For transition induction, we will selectively stimulate identified "switch ensembles" at random times to test if artificial activation is sufficient to trigger a spontaneous transition between actions (Fig. 1E, right).

Technical implementation: We will primarily use spiral scanning (spiraling the laser focus over the cell body for ~10-20 ms) to generate robust bursts of action potentials in targeted cells (Russell et al., 2022). We will incrementally increase the number of simultaneously stimulated neurons to determine the minimum number of co-active neurons necessary to trigger a behavioral switch. We will also vary the timing of stimulation relative to the trial phase (early vs. late in a foraging bout). Because there are tradeoffs

between the speed and power of the stimulation and the number of neurons one can stimulate (Sridharan et al., 2022), the choice of the opsin will depend on the nature of the neuronal patterns at hand (see Part 3. Implementation for more details).

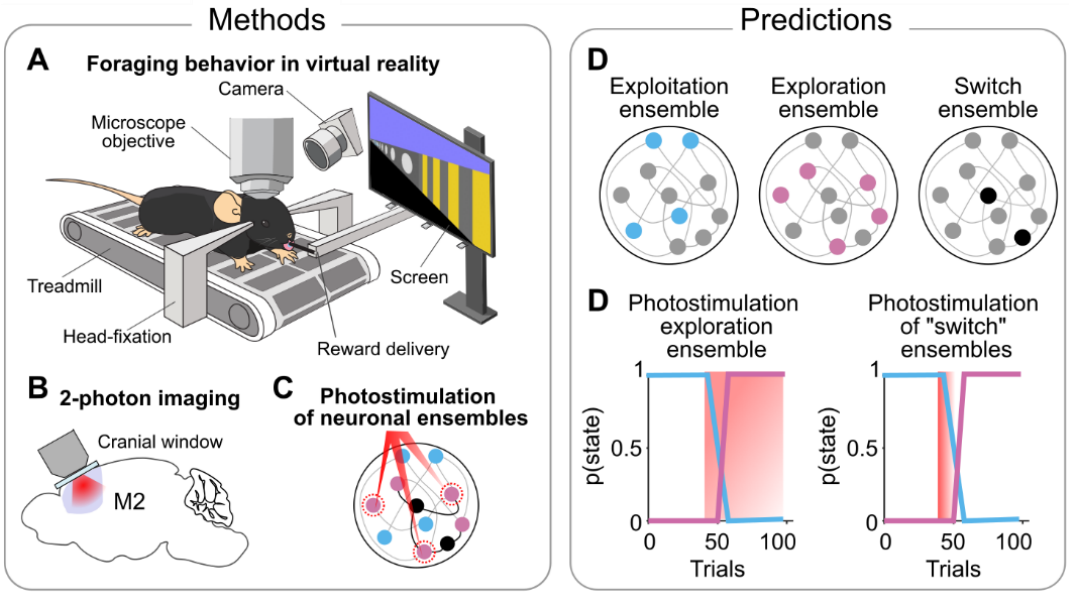

Figure 1. Methods and predictions. A. A mouse exploits a virtual resource site (materialized color stripes on the screen). A trial consists of a series of rewarded and unrewarded licks at a spout attached to a movable arm. Independently from site depletion, mice can choose to leave the site at any time by running a set distance on the treadmill to explore whether there is a more rewarding site elsewhere. During this exploration phase, the arm moves away and then moves back into place, while the animal travels along a virtual corridor (grey windows on the screen). The mouse can then forage at a new site. **B.** Illustration of 2P calcium imaging of M2 neurons. **C.** Targeted 2P photostimulation of different ensembles during the task. **D.** Identification of different neuronal ensembles associated with different behavioral states. **E.** Effect of 2P photostimulation on behavioral states. Sustained stimulation of the exploration ensemble favors running (left). Transient stimulation of the switch ensembles induces an abrupt transition in behavioral states and mice switch from licking to running (right).

Gender dimension in research content: In the field of neuroscience, the design of animal experiments has long been driven by outdated gender stereotypes, namely that the female reproductive physiology significantly influences brain processing (Shansky, 2019). As a result, it is still common practice to work with male animals exclusively and sex differences are understudied. Here, just like in our previous studies (Cazettes et al., 2025, 2023, 2021), we will use mixed-sex cohorts. Prior to pooling data for analysis, we will systematically test for significant differences between sexes in behavioral performance (e.g., foraging efficiency, transition frequency, response to stimulation) and neural activity patterns (e.g., ensemble composition, subspace geometry, transition dynamics). If significant sex differences are identified, we will analyze male and female cohorts separately and report sex-specific findings. This approach ensures that our conclusions are not biased by undetected sex effects and contributes to addressing the historical underrepresentation of female subjects in neuroscience research.

1.2. Interdisciplanary and intersectoral dimension of the project

Interdisciplinary nature and complementarity of the partners: This project is inherently interdisciplinary, integrating expertise from multiple scientific domains:

- Systems neuroscience & behavior: The host laboratory (Dr. Cazettes, INT Marseille) provides expertise in behavioral task design for studying foraging decisions and large-scale electrophysiology. The PhD student will be trained in cutting-edge behavioral paradigms and neural data acquisition.

- Biophotonics & optogenetics: The co-supervisor (Dr. Tommaso Fellin, IIT Genova, Italy) is a world-leading expert in all-optical interrogation techniques. His laboratory has pioneered the development of optical methods for simultaneous imaging and manipulation, including holographic 2P stimulation combined with calcium imaging (Forli et al., 2021, 2018; Dal Maschio et al., 2010). The secondment at IIT will provide hands-on training in these cutting-edge optical techniques.

- Computational neuroscience: The project requires computational methods for analyzing neural population dynamics, identifying neuronal ensembles, and for building computational models of decision-making. Analysis will be supervised by Dr. Cazettes, who has expertise in computational modeling of decision-making and in analysis of high-throughput neural recording. The candidate will also be supported by a dedicated computational engineer in the host team and benefit from collaboration with Dr. Cazettes' close theory collaborator: Dr. Luca Mazzucato (University of Oregon), an expert in theoretical modeling of neural dynamics. This interdisciplinary training will prepare the student for the increasingly computational nature of modern neuroscience.

Hosting arrangements and international dimension: The project involves a structured international collaboration between France and Italy. The **primary host** is the Institut de Neurosciences de la Timone (INT), CNRS/Aix-Marseille University, France, with Dr. Fanny Cazettes. The student will conduct the majority of their research here, including behavioral experiments, calcium imaging, and data analysis. The **international secondment** (3-6 months) will be at the Istituto Italiano di Tecnologia (IIT), Genova, Italy, with Dr. Tommaso Fellin. The student will receive intensive training in imaging and all-optical interrogation techniques, including holographic 2P stimulation setup and protocols for combining imaging with optogenetic manipulation in behaving mice. Dr. Fellin's laboratory has extensive experience with various opsin-indicator combinations and has developed strategies to maximize stimulation efficiency while minimizing photodamage and cross-talk. The secondment can be split into two periods (e.g., 3+3 months) with back-and-forth travel, allowing the student to learn optical protocols at IIT, attempt implementation at INT, and return to IIT for troubleshooting and refinement if needed.

Intersectoral dimension: The project includes a non-academic partnership with **Bruker**, a leading manufacturer of advanced microscopy systems and an established partner of the **Equipex CircuitPhotonics** program, which is part of the imaging platform at the host institution. Bruker manufactures the Ultima 2Pplus microscope used at INT, which has been upgraded with a Spatial Light Modulator (SLM, acquired by Dr. Cazettes) enabling holographic two-photon stimulation. Here, this partnership goes beyond equipment provision and involves direct scientific–technical interactions with the PhD student.

Bruker engineers will interact regularly with the student through **scheduled video sessions** dedicated to training on microscope operation, SLM control, and development of stimulation protocols (e.g. targeting specific neuronal patterns at defined behavioral time points and interfacing the microscope with the behavioral setup). In addition, **on-site visits by Bruker technical staff**, for example during laser alignment, system calibration, and early stimulation experiments, will provide hands-on exposure to optical system integration and troubleshooting. These interactions are expected to occur primarily during the setup and optimization phases of the project.

Through this collaboration, the student will gain: (1) practical training in optical system engineering, SLM alignment, and performance optimization; (2) experience contributing user feedback to the development of next-generation all-optical microscopy systems; and (3) exposure to industry R&D practices, quality constraints, and career opportunities outside academia.

The involvement of Bruker as a scientific and technical partner, reinforced by prior collaborations with Dr. Fellin on optical methods for neural interrogation, ensures meaningful intersectoral training and strengthens the translational dimension of the project.

Added value of the consortium: This unique combination of expertise is essential for project success. Studying neural ensemble dynamics during action selection requires: sophisticated behavioral paradigms and analysis (Cazettes), cutting-edge all-optical techniques (Fellin), and understanding of microscopy systems (Bruker) with local technical support from a dedicated research engineer at INT (A. Lombardini). The project demands advanced computational skills for analyzing terabyte-scale imaging datasets, implementing ensemble detection algorithms, applying dimensionality reduction methods, and building dynamical models (Cazettes & Mazzucato). It is rare to find a single laboratory possessing all these capabilities, making the collaborative structure essential. The PhD student will emerge with a rare combination of skills spanning behavior, imaging, optogenetics, computational analysis, and technology development, highly valuable for both academic and industry careers in the growing field of neurophotonics.

2. IMPACT (2 pages max)

2.1. Expected impact of the project on the candidate's career

This PhD program will provide comprehensive training that positions the candidate for leadership in systems neuroscience or related industry sectors. The combination of international mobility, interdisciplinary research, and intersectoral exposure ensures a well-rounded professional development.

Scientific skills acquired:

- Behavioral neuroscience: Implementing sophisticated behavioral paradigms for studying foraging decisions in head-fixed mice, including virtual reality environments and multi-modal behavioral tracking.

- High-throughput 2P calcium imaging: Longitudinal recording of large neural populations across weeks, including surgical procedures (cranial window implantation), viral injections for calcium indicator expression (GCaMP8), and chronic imaging protocols.

- All-optical interrogation: Holographic 2P photostimulation with single-cell resolution, including SLM programming, opsin selection and expression strategies, and crosstalk minimization between imaging and stimulation wavelengths.

- Opsin expression strategies: Viral delivery (AAV vectors) and transgenic approaches for co-expression of calcium indicators and opsins, including optimization of expression levels and cell-type specificity.

- Computational analysis: Methods for neural population dynamics including dimensionality reduction, clustering algorithms for ensemble identification, and dynamical systems analysis of neural trajectories.

Transferable skills:

Through the intersectoral partnership and training program, the candidate will develop: **project management** (coordinating experiments and managing timelines), **science communication** (presenting at international conferences and writing for peer-reviewed journals), **industry collaboration** (working with Bruker on technology development and user feedback), and **international networking** (building connections across European research institutions).

Career prospects:

- Academic positions: Postdoctoral researcher and eventually principal investigator in systems neuroscience, with expertise in cutting-edge optical techniques that are increasingly in demand.

- Industry positions: R&D scientist or applications specialist at biophotonics/neurotechnology companies (e.g., Light Conversion, Bruker, 3i, Thorlabs), leveraging on expertise in advanced microscopy and optical manipulation.

- Interface positions: Technology transfer offices, science policy, or science communication roles that benefit from deep technical expertise combined with broad interdisciplinary training.

2.2. Expected impact for the thematic axis

Scientific impact:

This project will advance our understanding of how neural circuits implement flexible behavior, a fundamental question in neuroscience with broad implications. Key expected contributions include:

- First causal demonstration that targeted activation of neural ensembles can bias action selection during foraging decisions, establishing the functional role of M2 ensemble activity in real-time behavioral control.
- Identification of 'switch ensembles' that trigger transitions between behavioral states, potentially revealing a general neural mechanism for behavioral flexibility.
- Methodological advances in combining longitudinal imaging with targeted 2P photostimulation for studying behavioral flexibility, providing protocols that can be adopted by other laboratories.
- New insights into the neural subspace organization of motor representations, contributing to theoretical frameworks of neural computation.

Societal and translational impact on "*health and well-being*":

Inability to flexibly switch between actions underlies numerous psychiatric and neurological disorders:

- **Depression:** Characterized by difficulty initiating beneficial actions and excessive perseveration on negative thoughts.
- **Addiction and compulsion:** Marked by inability to refrain from detrimental actions despite awareness of negative consequences.
- **Age-related cognitive decline:** Often manifests as reduced cognitive flexibility and difficulty adapting to changing circumstances.
- **Parkinson's disease:** Motor inflexibility and difficulty initiating or switching between movements.

Understanding the neural circuit mechanisms of flexible action selection may reveal biomarkers for individual differences in decision-making abilities, potential therapeutic targets for disorders of behavioral inflexibility, and principles for developing more flexible artificial intelligence systems inspired by biological neural circuits.

2.3. Dissemination, exploitation and communication activities planned

Scientific dissemination:

- **Publications:** Target of 1-2 first-author publications in high-impact peer-reviewed journals (e.g., Nature Neuroscience, Neuron, Cell Reports). Methodology papers may also be submitted to technical journals (e.g., Nature Methods, eLife).
- **Conference presentations:** Annual presentations at major international conferences including the Society for Neuroscience (SfN), Federation of European Neuroscience Societies (FENS), and Computational and Systems Neuroscience (Cosyne).
- **Open science practices:** Sharing of analysis code on GitHub, datasets on Figshare or DANDI archive, and preprints on bioRxiv prior to peer review. All analysis pipelines will be documented to facilitate reproducibility.

Public engagement:

- **Science festivals:** Participation in Brain Awareness Week events or Pints of Science, presenting research to the general public through interactive demonstrations.
- **Science communication:** Blog posts explaining research findings for non-specialist audiences, social media presence (LinkedIn, Bluesky) to share scientific progress.
- **Student engagement:** Presentations to university students to develop interest in neuroscience research careers.

Exploitation: Methodological developments, particularly protocols for combining longitudinal imaging with holographic stimulation, will be documented in detail and shared to enable adoption by other research groups. The industry partnership with Bruker will explore potential applications of the research for technology development, including user feedback for improving next-generation all-optical systems and potential patent applications for novel optical configurations.

3. IMPLEMENTATION (2 pages max)

3.1. Work plan

Year 1: Establishing foundations and imaging

Month 1: Welcome to INT Marseille. Registration for animal work (consolidator formation + surgery formation). Intensive observation, literature review on all-optical methods and neural population dynamics.

Months 2-4: First secondment at IIT Genova (Dr. Fellin). Intensive training in: (1) 2P calcium imaging protocols, (2) Holographic 2P stimulation setup, (3) Strategies to minimize crosstalk between imaging and stimulation wavelengths, (4) Surgical procedures (cranial window implantation) and viral injections for calcium indicator expression (GCaMP8).

Months 5-6: Return to INT. Intensive training in the foraging task paradigm (flipping task), mouse handling, and behavioral training protocols.

Months 7-12: 2P imaging experiments. Longitudinal calcium imaging of M2 neurons during foraging behavior. Focus on identifying neural activity patterns associated with licking (exploit) versus running (explore) actions. Development of analysis pipelines for ensemble identification in collaboration with engineers in the team.

Year 2: All-optical setup and pilot experiments

Months 13-15 (if necessary): Second secondment at IIT Genova (Dr. Fellin). Troubleshooting from year 1: SLM optimization and holographic pattern generation, stimulation protocol design for behavioral experiments, etc. Parallel: continued computational analysis of imaging data from Year 1.

Months 16-24: Return to INT. Implementation of SLM-based holographic stimulation on the Ultima 2Pplus microscope. Validation of all-optical protocols in head-fixed mice. Pilot stimulation experiments targeting identified ensembles to optimize parameters.

Year 3: Causal experiments and synthesis

Months 25-32: Systematic all-optical manipulation experiments: (1) Stimulation of 'exploit ensembles' to bias toward staying/licking, (2) Stimulation of 'explore ensembles' to bias toward leaving/running, (3) Stimulation of 'switch ensembles' to trigger behavioral transitions, (4) Parametric exploration: number of neurons, timing, and temporal patterns required to influence behavior (if time allows).

Months 33-36: Final data analysis and integration. Manuscript preparation. PhD thesis writing and defense preparation.

Intersectoral collaboration with Bruker: Rather than a formal secondment, interaction with Bruker will be structured as ongoing technical exchange throughout the project, with highest intensity in Years 1–2 during implementation and optimisation of SLM-based holographic two-photon stimulation on the Ultima 2Pplus at INT. The student will interact regularly with Bruker application specialists through scheduled video calls and targeted support during key milestones (e.g., alignment, calibration, early stimulation experiments) to troubleshoot and optimise optical performance and integration with the behavioral setup. This collaboration provides hands-on training in optical system engineering, exposure to industry R&D and quality constraints, and a channel for structured user feedback contributing to next-generation all-optical system development.

Milestones and deliverables:

- **M1 (Month 6):** Completion of training at IIT and first cohort of mice implanted with cranial windows and viral injections at INT. *Deliverable:* Technical report on 2P imaging and 2P stimulation protocols.
- **M2 (Month 12):** First imaging dataset acquired and analyzed. *Deliverable:* Identification of exploit/explore neural ensembles and preliminary subspace analysis.
- **M3 (Month 15):** All-optical protocol validation completed. *Deliverable:* Working all-optical setup with demonstrated near single-cell stimulation accuracy.

- **M4 (Month 24):** Pilot 2P stimulation experiments completed. *Deliverable:* Proof-of-concept data showing behavioral effects of ensemble stimulation.
- **M5 (Month 32):** Full causal dataset acquired. *Deliverable:* Complete analysis of stimulation effects on action selection and transitions.
- **M6 (Month 36):** PhD thesis defense. *Deliverables:* PhD thesis, 1-2 manuscripts submitted, analysis code repository.

Feasibility and risk mitigation:

- Operational readiness: The project incurs zero "start-up" latency. The behavioral setup under the microscope is operational and calibrated. Preliminary data demonstrates high-quality 2P calcium imaging of M2 neurons in mice running on the treadmill (Fig. 2). Feasibility is further strengthened by dedicated on-site support from Dr. Lombardini, a research engineer at INT and member of the imaging platform, who will assist with daily microscope operation and optimisation and is already closely connected with Bruker application specialists.

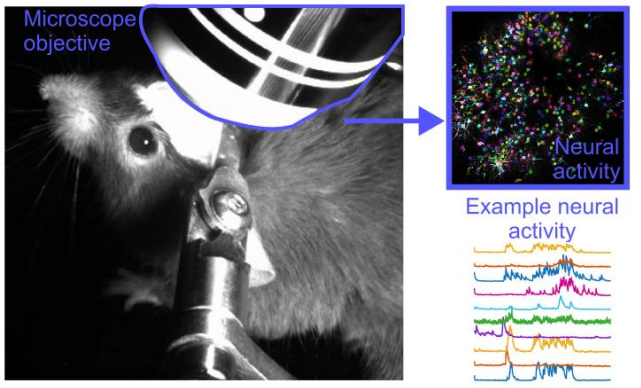

Figure 2. Preliminary data. A mouse walks on a treadmill while neural activity is recorded from ~300 M2 neurons using 2P calcium imaging through a cranial window. Some examples extracted traces are displayed on the right.

- Administrative efficiency: To prevent delays associated with mandatory French animal experimentation training, expedited private training sessions can be arranged if standard university sessions are unavailable.

- Opsin selection and crosstalk: rsChRmine (Kishi et al., 2022) will be prioritized for its high sensitivity and large conductance, allowing simultaneous activation of larger ensembles (~30-50 neurons) with lower laser power, reducing the risk of tissue heating (Picot et al., 2018). If crosstalk between 920 nm imaging and 1030 nm stimulation proves problematic, we will test ChrimsonR (lower conductance but better spectral separation (Klapoetke et al., 2014)), increase expression level of GCaMP8, decrease dwell time and number of pixels per neuronal ROI, and tune our imaging beam at shorter wavelength (900 nm).

- Depth of stimulation: M2 action representations are distributed across cortical layers. While all-optical 2P stimulation is most efficient in superficial layers (L2/3, <400 µm), the high power of the CARBIDE laser (40W average, ~40 µJ/pulse) combined with ChRmine's excellent 2P cross-section enables stimulation at depths up to 800 µm (Layer 5). Studies in visual cortex have shown that stimulating small numbers of L5 neurons can be as behaviorally effective as larger L2/3 ensembles due to their strong output connectivity (Marshel et al., 2019b).

- Ensemble stability: Neural representations may drift across days. To account for this, we will perform intermittent "read" imaging sessions to track ensemble stability and update stimulation patterns accordingly. The SLM allows rapid reconfiguration of holographic patterns (<100 ms) to target the most current functional ensembles identified in recent imaging sessions.

- Independent objectives: The imaging experiments (Objectives 1-2) do not depend on the success of all-optical stimulation, ensuring meaningful results even if stimulation proves challenging. Dr. Fellin's extensive expertise in all-optical methods greatly reduces technical risk for Objective 3.

Supervision and mentoring: The PhD student will benefit from weekly supervision meetings at INT with Dr. Cazettes and her team, ongoing interaction with the international co-supervisor (Dr. Fellin) through regular video conferences and secondments, annual thesis committee meetings with external experts, direct support from optical engineer (A. Lombardini, INT) and data engineer (A. Aberlenc, Cazettes team), and collaboration with Dr. Luca Mazzucato (University of Oregon) for computational analysis support.

4. REFERENCES

Carrillo-Reid, L., Han, S., Yang, W., Akrouh, A., Yuste, R., 2019. Controlling Visually Guided Behavior by Holographic Recalling of Cortical Ensembles. Cell 178, 447-457.e5. https://doi.org/10.1016/j.cell.2019.05.045

Carrillo-Reid, L., Yang, W., Bando, Y., Peterka, D.S., Yuste, R., 2016. Imprinting and recalling cortical ensembles. Science 353, 691–694. https://doi.org/10.1126/science.aaf7560

Cazettes, F., Mazzucato, L., Murakami, M., Morais, J.P., Augusto, E., Renart, A., Mainen, Z.F., 2023. A reservoir of foraging decision variables in the mouse brain. Nat. Neurosci. 26, 840–849. https://doi.org/10.1038/s41593-023-01305-8

Cazettes, F., Reato, D., Augusto, E., Steinfeld, R., Renart, A., Mainen, Z.F., 2025. Facial expressions in mice reveal latent cognitive variables and their neural correlates. Nat. Neurosci. 28, 2310–2318. https://doi.org/10.1038/s41593-025-02071-5

Cazettes, F., Reato, D., Morais, J.P., Renart, A., Mainen, Z.F., 2021. Phasic Activation of Dorsal Raphe Serotonergic Neurons Increases Pupil Size. Curr. Biol. CB 31, 192-197.e4. https://doi.org/10.1016/j.cub.2020.09.090

Forli, A., Pisoni, M., Printz, Y., Yizhar, O., **Fellin, T**., 2021. Optogenetic strategies for high-efficiency all-optical interrogation using blue-light-sensitive opsins. eLife 10, e63359. https://doi.org/10.7554/eLife.63359

Forli, A., Vecchia, D., Binini, N., Succol, F., Bovetti, S., Moretti, C., Nespoli, F., Mahn, M., Baker, C.A., Bolton, M.M., Yizhar, O., **Fellin, T**., 2018. Two-Photon Bidirectional Control and Imaging of Neuronal Excitability with High Spatial Resolution In Vivo. Cell Rep. 22, 3087–3098. https://doi.org/10.1016/j.celrep.2018.02.063

Gill, J.V., Lerman, G.M., Zhao, H., Stetler, B.J., Rinberg, D., Shoham, S., 2020. Precise Holographic Manipulation of Olfactory Circuits Reveals Coding Features Determining Perceptual Detection. Neuron 108, 382-393.e5. https://doi.org/10.1016/j.neuron.2020.07.034

Inagaki, H.K., Fontolan, L., Romani, S., Svoboda, K., 2019. Discrete attractor dynamics underlies persistent activity in the frontal cortex. Nature 566, 212–217. https://doi.org/10.1038/s41586-019-0919-7

Kishi, K.E., Kim, Y.S., Fukuda, M., Inoue, M., Kusakizako, T., Wang, P.Y., Ramakrishnan, C., Byrne, E.F.X., Thadhani, E., Paggi, J.M., Matsui, T.E., Yamashita, K., Nagata, T., Konno, M., Quirin, S., Lo, M., Benster, T., Uemura, T., Liu, K., Shibata, M., Nomura, N., Iwata, S., Nureki, O., Dror, R.O., Inoue, K., Deisseroth, K., Kato, H.E., 2022. Structural basis for channel conduction in the pump-like channelrhodopsin ChRmine. Cell 185, 672-689.e23. https://doi.org/10.1016/j.cell.2022.01.007

Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., Wang, J., Xie, Y., Yan, Z., Zhang, Y., Chow, B.Y., Surek, B., Melkonian, M., Jayaraman, V., Constantine-Paton, M., Wong, G.K.-S., Boyden, E.S., 2014. Independent optical excitation of distinct neural populations. Nat. Methods 11, 338–346. https://doi.org/10.1038/nmeth.2836

Kolling, N., Behrens, T.E.J., Mars, R.B., Rushworth, M.F.S., 2012. Neural Mechanisms of Foraging. Science 336, 95–98. https://doi.org/10.1126/science.1216930

Marques, J.C., Li, M., Schaak, D., Robson, D.N., Li, J.M., 2020. Internal state dynamics shape brainwide activity and foraging behaviour. Nature 577, 239–243. https://doi.org/10.1038/s41586-019-1858-z

Marshel, J.H., Kim, Y.S., Machado, T.A., Quirin, S., Benson, B., Kadmon, J., Raja, C., Chibukhchyan, A., Ramakrishnan, C., Inoue, M., Shane, J.C., McKnight, D.J., Yoshizawa, S., Kato, H.E., Ganguli, S., Deisseroth, K., 2019a. Cortical layer–specific critical dynamics triggering perception. Science 365, eaaw5202. https://doi.org/10.1126/science.aaw5202

Marshel, J.H., Kim, Y.S., Machado, T.A., Quirin, S., Benson, B., Kadmon, J., Raja, C., Chibukhchyan, A., Ramakrishnan, C., Inoue, M., Shane, J.C., McKnight, D.J., Yoshizawa, S., Kato, H.E., Ganguli, S., Deisseroth, K., 2019b. Cortical layer–specific critical dynamics triggering perception. Science 365, eaaw5202. https://doi.org/10.1126/science.aaw5202

Dal Maschio, M, Difato, F., Beltramo, R., Blau, A., Benfenati, F., **Fellin, T**., 2010. Simultaneous two-photon imaging and photo-stimulation with structured light illumination. Opt. Express 18, 18720–18731. https://doi.org/10.1364/OE.18.018720

Picot, A., Dominguez, S., Liu, C., Chen, I.-W., Tanese, D., Ronzitti, E., Berto, P., Papagiakoumou, E., Oron, D., Tessier, G., Forget, B.C., Emiliani, V., 2018. Temperature Rise under Two-Photon Optogenetic Brain Stimulation. Cell Rep. 24, 1243-1253.e5. https://doi.org/10.1016/j.celrep.2018.06.119

Recanatesi, S., Pereira-Obilinovic, U., Murakami, M., Mainen, Z., Mazzucato, L., 2022. Metastable attractors explain the variable timing of stable behavioral action sequences. Neuron 110, 139-153.e9. https://doi.org/10.1016/j.neuron.2021.10.011

Robinson, N.T.M., Descamps, L.A.L., Russell, L.E., Buchholz, M.O., Bicknell, B.A., Antonov, G.K., Lau, J.Y.N., Nutbrown, R., Schmidt-Hieber, C., Häusser, M., 2020. Targeted Activation of Hippocampal Place Cells Drives Memory-Guided Spatial Behavior. Cell 183, 1586-1599.e10. https://doi.org/10.1016/j.cell.2020.09.061

Russell, L.E., Dalgleish, H.W.P., Nutbrown, R., Gauld, O.M., Herrmann, D., Fişek, M., Packer, A.M., Häusser, M., 2022. All-optical interrogation of neural circuits in behaving mice. Nat. Protoc. 17, 1579–1620. https://doi.org/10.1038/s41596-022-00691-w

Shansky, R.M., 2019. Are hormones a “female problem” for animal research? Science 364, 825–826. https://doi.org/10.1126/science.aaw7570

Shin, H., Ogando, M.B., Abdeladim, L., Jagadisan, U.K., Durand, S., Hardcastle, B., Belski, H., Cabasco, H., Loefler, H., Bawany, A., Wilkes, J., Nguyen, K., Suarez, L., Johnson, T., Han, W., Ouellette, B., Grasso, C., Swapp, J., Ha, V., Young, A., Caldejon, S., Williford, A., Groblewski, P.A., Olsen, S., Kiselycznyk, C., Lecoq, J., Adesnik, H., 2025. Recurrent pattern completion drives the neocortical representation of sensory inference. Nat. Neurosci. 28, 2319–2329. https://doi.org/10.1038/s41593-025-02055-5

Sridharan, S., Gajowa, M.A., Ogando, M.B., Jagadisan, U.K., Abdeladim, L., Sadahiro, M., Bounds, H.A., Hendricks, W.D., Turney, T.S., Tayler, I., Gopakumar, K., Oldenburg, I.A., Brohawn, S.G., Adesnik, H., 2022. High-performance microbial opsins for spatially and temporally precise perturbations of large neuronal networks. Neuron 110, 1139-1155.e6. https://doi.org/10.1016/j.neuron.2022.01.008

Tafazoli, S., Bouchacourt, F.M., Ardalan, A., Markov, N.T., Uchimura, M., Mattar, M.G., Daw, N.D., Buschman, T.J., 2025. Building compositional tasks with shared neural subspaces. Nature 1–9. https://doi.org/10.1038/s41586-025-09805-2

5. ETHICS

5.1 Animal numbers and experimental procedures

This project will use approximately **40-50 mice** (Mus musculus) over three years:

- **Imaging (Year 1):** 20 mice for longitudinal 2P calcium imaging (including testing)
- **Photostimulation:** 20-30 mice for all-optical manipulation (including testing)

These numbers account for ~70% success rate considering surgical outcomes, viral expression efficiency, and behavioral training success.

Procedures include:

- Cranial window implantation over motor cortex (M2) and head-bar fixation under isoflurane anesthesia
- Viral injection for calcium imaging and optogenetic manipulation
- Behavioral training on head-fixed foraging task (3-4 weeks, 1-2 hours/session)
- 2P imaging and holographic 2P photostimulation during behavior (1-2 weeks)
- Euthanasia under deep anesthesia followed by perfusion for histology

5.2 Species rationale

Mus musculus is essential because:

- Mice perform complex decision-making tasks requiring strategic switching between actions
- Genetic tractability enables targeted expression of calcium indicators and opsins
- Compact brain size permits multi-layer cortical access with 2P microscopy
- Head-fixation protocols are well-established, enabling stable chronic optical access
- Mouse motor cortex shares organizational principles with other mammals
- No alternative exists: neural ensemble dynamics during flexible behavior cannot be replicated in vitro, computationally, or in invertebrate models lacking mammalian cortical architecture

5.3 Animal welfare measures

Daily monitoring: Animals are examined daily for body weight (≥85% baseline), hydration, coat condition, locomotor activity, wound healing, and behavioral responsiveness. Animals showing welfare concerns receive veterinary care or are euthanized.

Perioperative care: Pre-operative analgesia (buprenorphine 0.05-0.1 mg/kg, local lidocaine), continuous monitoring during surgery (respiration, temperature maintained at 37°C) and post-operative recovery on heating pads with additional analgesia (buprenorphine) and anti-inflammatory drugs (carprofen 5 mg/kg) for 48-72 hours

Behavioral sessions: Limited to 1-2 hours. Animals voluntarily control participation by initiating licking.

Water management: We implement a refined **citric acid water protocol**: mice have free access to sour-tasting water in home cages (2% citric acid), naturally reducing intake while maintaining healthy weight (≥85% baseline) and motivation for sweet rewards. This minimizes stress compared to complete restriction. Natural water provided freely on non-training days. Any animal losing >15% weight receives immediate free water access.

Housing: Enriched environments with nesting material, tunnels, shelters; 12-hour light/dark cycle; temperature-controlled rooms (20-24°C); social housing when compatible with experiments.

Humane endpoints: Animals removed from study if: sustained weight loss >20%, infection unresponsive to treatment, neurological deficits, self-injury to surgical sites, or chronic distress indicators.

5.4 The 3Rs Principle

Replacement: Complete replacement is not feasible. Neural ensemble dynamics controlling flexible action selection involve distributed cortical networks that cannot be replicated in vitro or simulated. Understanding how neural activity causally drives naturalistic behavior requires intact, behaving animals making voluntary decisions. The mammalian motor cortex organization is conserved across species, making mouse findings translationally relevant.

Reduction: We minimize numbers through:

- Power analysis based on preliminary data ensuring adequate statistical power

- Within-subject longitudinal designs tracking same neurons across sessions
- High-density 2P imaging (>1000 neurons simultaneously) maximizing data per animal
- Interleaved control/stimulation trials within sessions, eliminating separate control cohorts
- Public data sharing enabling secondary analyses, reducing redundant experiments
- Small pilot cohorts (N=3-5) optimizing protocols before large-scale studies

Refinement: Pain and stress minimized through:

- Minimally invasive surgical techniques with multimodal analgesia (local and systemic)
- Voluntary task participation: animals control session progression
- Citric acid water protocol reducing restriction stress
- Gradual habituation to head-fixation before experiments
- Environmental enrichment (social housing, nesting materials, tunnels)
- Laser powers calibrated below thermal/photodamage thresholds
- Gentle handling, consistent experimenter, predictable routines

5.5 Ethical oversight and compliance

All procedures comply with European Directive 2010/63/EU, European Convention ETS No. 123, French regulations, and Commission Recommendation 2007/526/EC.

Institutional framework: Research conducted at Institut de Neurosciences de la Timone (INT) with CNRS-accredited facilities (Agreement N° B1301404) under institutional veterinarian supervision (Dr. I. Balansard). All protocols require approval by Laurent Vinay Ethics Committee (CEEA 71) via national APAFIS system before initiation.

Personnel: All personnel hold required accreditations. The PhD candidate will complete mandatory training and work under PI supervision until obtaining independent authorization.

Biosafety: Viral vectors handled following BSL-1 protocols. Waste disposal follows certified procedures complying with European Directive 2008/98/EC.

No dual-use concerns: This fundamental neuroscience research has no military or security applications.

5.6 Justification and impact

This research requires invasive techniques incompatible with human subjects, but addresses mechanisms underlying disorders affecting millions: obsessive-compulsive disorder, addiction, and depression all involve impaired behavioral flexibility. Identifying how neural ensembles control action switching may inform precision neuromodulation therapies (e.g., closed-loop deep brain stimulation) restoring adaptive flexibility in patients. The mouse model provides essential mechanistic insights and proof-of-principle for future clinical applications while maintaining highest ethical standards for animal welfare.