**Application number: 324/24**

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**Neural control of social decisions in naturalistic social environment**

**scientific abstract**

Social decisions are critical to human prosperity and suboptimal social decision-making is associated with varied psychiatric and neurological disorders. Whereas significant progress has been made in deciphering the neural basis of social decision-making in animal models and simplified lab tasks, social decisions in natural environments are typically more complex. From behavioral responses to social cues to the selection of a mating partner, social decisions in the wild are context dependent, relying on multiple factors such as group size and heterogeneity, animal density, the level of competition and the effect of other recent social encounters.

Here, we propose to better understand the neural basis of naturalistic social decisions using the vinegar fly, *Drosophila melanogaster*, as a model system. We have two hypotheses. First, that social decisions in both sexes depend on social context, including decisions who directly involve two flies (e.g., male choosing the next song note or female responding to a copulation attempt) and decisions who involve more flies (e.g., male switching from one to another female target). Second, that these decisions, within the social context, are modulated by a specific set of sexually dimorphic cells in the central brains of male and female flies.

Accordingly, this proposal has two aims. First, we aim to reveal how immediate multisensory sensory cues (visual, auditory, and tactile), social context (specifically, male-female ratio) and recent social experiences (e.g., recent rejections or fights) determine male decisions including song choice, initiating/terminating courtship or switching target and female decision to accept or reject a copulation attempt. Second, we aim to determine how the activity of sexually dimorphic cells, in male and female central brains, modulate these social decisions.

We will take advantage of new tools for fine behavioral quantification of social behavior and the ability to apply optogenetic stimulation to well defined neural populations in intact, freely moving flies. Using modern recording methods, we will measure visual, auditory, and tactile stimuli in our environments, as flies behave, and we will computationally estimate of how these stimuli are experienced by individuals, in their own body’s perspective. We will analyze the data to assess whether complex social decisions can be predicted by a combination of the immediate sensory inputs, group dynamics and recent experience of the deciding fly. To assess how the activity of central neurons modulates social decisions we will use both open- and closed- loop optogenetic experiments, measuring the effects of tonic and phasic activation of neurons during social behaviors. Overall, this work should allow us to develop a new, and more comprehensive framework for understanding the neural basis of social decision making in a tractable species. Our goal is to make the behavior of individuals in complex social environments increasingly sensible, building on the more reductionistic experiments of the past alongside the modern recording and analysis methods of the present.

**Detailed description of the research program**

**POINTS to add in the background-**

1. The female perspective. Females respond by slowing, turning, OE, and a set of rejection behaviors (accept/reject copulation). Also - maybe - in turning towards another male. In dyads seems like males keep courting regardless of the female response. But is it true when they have options? does it change between their first and later attempts (e.g., when realizing than there are no better options).

the terms no-choice; choice; true-choice. Females prefer large and mature males.

<https://www.sciencedirect.com/science/article/pii/S0003347218300605?casa_token=aXK5bxXSkbEAAAAA:QpXmKKP2_h1LQep0VNqRVXpeJs0yP5OG5ot5Tpjw-nkVrLZV8ds0H77Dkvj28CR-gtJI3ekIiw#bib17>

mating speed was discussed in multiple studies. Some say that it is important (<https://link.springer.com/article/10.1007/BF01066159> ) while others say that it is not (<https://link.springer.com/article/10.1007/BF01067436> ).

1. **Scientific background**

Social communication is essential for our survival and well-being. Interpersonal communication is complex and state-dependent: it relies on the integration of dynamically changing multisensory cues, as well as on the needs and the physiological state of the participants. Accordingly, many brain disorders are characterized by deficits in social integrations [1](https://paperpile.com/c/cMiCci/i7qd).

Studying social communication in animal model systems has been beneficial for the understanding of the processing and production of social cues [2–4](https://paperpile.com/c/cMiCci/d8Yv+B2mP+i4zI). While the importance of studying animal behaviors in their natural habitats has been proven critical in multiple studies, lab experiments, particularly neurobiology-oriented ones, are often conducted in highly simplified settings unlikely to reflect the conditions under which the underlying neural circuits evolved. Revealing the neural basis of social decisions in complex environments requires both computational tools for tracking and analyzing the behavior of multiple interacting individuals, as well as experimental tools for temporal manipulations of specific neural circuits in intact and freely behaving animals.

Here we propose to tackle these problems by using the fruit fly *Drosophila melanogaster* as a model system, focusing on mating and aggressive behaviors in males and females. We take advantage of novel deep learning-based tools for multi-animal pose tracking and behavior segmentation, existing knowledge about the circuits underlying social behaviors, and our ability to temporally manipulate well-defined neural populations in intact flies using optogenetics.

First, we will establish a novel framework for the study of social communication in complex, naturalistic environments, and quantify how individual decisions and group dynamics are correlated with recent and past experience. Second, we will leverage these behavioral characterizations to reveal the role of specific sexually dimorphic neurons in controlling aggressive and mating behaviors, as well as the interactions between the two. Our work will pave the road for further experiments, where the neural basis of social communication is studied in ethologically relevant contexts.

**1.1 Social communication in *Drosophila***

*Drosophila melanogaster* flies show a variety of social behaviors, including mating and aggression [5](https://paperpile.com/c/cMiCci/16Xx). Using the available toolkit in this model system, neurobiologists revealed much of the circuit controlling mating and aggression in flies [6,7](https://paperpile.com/c/cMiCci/qjU7N+rLrL). While most neurobiology-oriented studies characterize mating behaviors in isolated male-female pairs on homogenous, food-free backgrounds [8–11](https://paperpile.com/c/cMiCci/dGlb1+TIxLC+awLRu+zESsQ), in their natural habitat flies aggregate densely on food patches, where they feed, fight and mate [12–14](https://paperpile.com/c/cMiCci/1BOOs+N0Yyf+TeKjH). Groups of flies exhibit non-random group structures and social interactions, which depend on multiple factors such as genetic heterogeneity [14–16](https://paperpile.com/c/cMiCci/TeKjH+DrUXD+mMg6q), group size and density [17](https://paperpile.com/c/cMiCci/h0zxg), the existence of rivals [18](https://paperpile.com/c/cMiCci/bqAEL), and sex ratio [19–21](https://paperpile.com/c/cMiCci/vcPY+Cg3Z+AtT6). Competition over a female partner depends not only on the male:female ratio, but also on the fraction of receptive females at any given moment, and the higher the ratio between motivated males to receptive females is, the more competition there is for a female partner [12,20](https://paperpile.com/c/cMiCci/Cg3Z+1BOOs). Social dynamics influence the timing of various behaviors in flies, from locomotor activity bouts to circadian patterns of mating [14,15,22,23](https://paperpile.com/c/cMiCci/TeKjH+DrUXD+xiRJP+vIdPN). Social decisions also depend on social experiences such as social isolation [24](https://paperpile.com/c/cMiCci/Iv07I), previous aggressive encounters [25](https://paperpile.com/c/cMiCci/mnQKc) and recent mating encounters [26](https://paperpile.com/c/cMiCci/M4le). Taken together, these findings support the notion that group living is a fundamental component of *Drosophila* behavior [14](https://paperpile.com/c/cMiCci/TeKjH). Social decisions such as choosing a mate or engaging in a fight depend on incoming sensory information about the prospect partner [6,27,28](https://paperpile.com/c/cMiCci/TIGWI+qjU7N+ndEg), but also on accumulated information about social environment and experience [16,29](https://paperpile.com/c/cMiCci/2cq11+mMg6q). Context-dependent social decisions are possible through spatial integration of sensory cues (e.g., olfactory cues about the density or heterogeneity of the group), and through temporal integration of previous events (such as recent mating or fighting), through their effect on the animals’ internal state (e.g., motivation or arousal), memory (e.g., a previous aggressive encounter) or direct changes in physiology (e.g., the injection of sex-peptide during mating [30,31](https://paperpile.com/c/cMiCci/XDIk+UFACz)).

A common framework for predicting and interpreting animal decisions (social or non-social) is ‘cost-benefit’ analysis. This framework is based on the assumption that nervous systems evolved to be somewhat optimized to the environment the organism evolved in. For example, remating in insects was shown to cause increased lifetime offspring production (benefit), while having a negative effect on female longevity (cost) [20](https://paperpile.com/c/cMiCci/Cg3Z). While we are able to predict some decision biases based on theory or past experiments, it is not trivial to predict moment-by-moment decisions based on theoretical considerations alone. For example, we may expect that a highly competitive environment for males will lead to more consistent courtship of a female, however, it is less clear how time spent courting a female versus showing aggression towards a competitor male in the vicinity will change as a function of the male:female ratio. We expect these types of decisions to occur frequently in nature where more animals are interacting freely. Indeed, in our preliminary data, we have found many instances of males interacting with a copulating pair, and of a male singing to a courting male in a male-male-female triplet (**Fig. 1**). Should these behaviors be interpreted as competitive or cooperative? To an extent, evaluating the costs and benefits depend on these interpretations. By reliably tracking the identity of individual flies over multiple hours and over a large number of behavioral events, and automatically detecting social behaviors (**Table 1**), we aim to reveal how social decisions are made in complex social environments (**Aim 1**), as well as test how these decisions, over multiple timescales, are modulated by the activation of neural populations who have high-level control of social behaviors in males and females (**Aim 2**).

**Table 1** Social behaviors in *Drosophila melanogaster.*

|  |  |  |
| --- | --- | --- |
| **Flies used for detecting the behavior** | **Reported behaviors** | **Selected references** |
| male | Singing (wing vibrations) | [10,32,33](https://paperpile.com/c/cMiCci/P8Oog+bcal+awLRu) |
| female | Ovipositor extrusion, vaginal plate opening, pausing, singing copulation song | [11,31,34–36](https://paperpile.com/c/cMiCci/yFOa+UFACz+nx6Vl+zESsQ+16HI) |
| male-female, female-male | Chasing, tapping, licking, copulating, circling  shoving (/fending), kicking, flicking, decamping | [6,9,37,38](https://paperpile.com/c/cMiCci/Co6t+qjU7N+yDW0+TIxLC) |
| male-male | chasing, singing, fighting (lunging, fencing, flicking) | [39–41](https://paperpile.com/c/cMiCci/9ef2n+w7TOO+ZoO5) |
| female-female | fighting (shoving, head-butting..) | [39,42](https://paperpile.com/c/cMiCci/9ef2n+bLfXM) |
| male-male-female | Male-male competition over a female  Male switching a female target\*  Male interacting with a mating pair\* | [43](https://paperpile.com/c/cMiCci/3q46) \* not characterized |
| female-female-male | Female interacting with a mating pair | [44](https://paperpile.com/c/cMiCci/WmGHl) |

**1.2 Quantifying social communication in complex environments**

Modeling social decisions in naturalistic environment based on the integration of complex sensory information (in time and space) and past experience (over multiple timescales) depends on the ability to track multiple individual flies at high spatiotemporal resolution to resolve their behavioral decisions and sensory experience (from the point of view of each individual fly, **Fig. 2**) over an extended period of time.

Motion capture technology enables the precise quantification of complex phenotypes from high-resolution videography of freely behaving animals [45](https://paperpile.com/c/cMiCci/yJI5I). Leveraging deep learning, the investigators’ previous work in developing these methods has recently been demonstrated to be feasible for tracking the motion of individual body parts of socially interacting animals, implemented using the SLEAP software framework [46](https://paperpile.com/c/cMiCci/CDwWB). SLEAP works by using deep neural networks that take video frames as an input and then predict the location of body parts, group them into animals, and link them across time to generate a continuous trajectory of poses for each animal. SLEAP has been found to be successful in tracking the poses of two animals for short periods of time (<1 h), with up to 99.9995% identity tracking accuracy (62 incorrect frames out of 11.7 million frames).

Reliable pose tracking allows us to extract features useful for inferring social behaviors. For example, we have found that wing angles/extensions can be used as a proxy for male singing (using a simple classifier we obtained 91% correct classification as to whether frames included part of a singing epoch), an important part of the male courtship ritual. The measurement of wing extension is also important for scoring aggressive behaviors, such as same-sex fights in males and females [39](https://paperpile.com/c/cMiCci/9ef2n) and rejection behaviors in unreceptive females [47,48](https://paperpile.com/c/cMiCci/KQXli+9aVsW). Finally, based on precise estimation of fly pose it is possible to estimate the sensory information (e.g., visual, auditory) that is available to the fly from the point of view of the animal (**Fig. 2**). Past work has shown that network models that consider the visual information accessible to each individual can be more predictive of behavior in collectives than those that don't incorporate this information [49](https://paperpile.com/c/cMiCci/h1VV).

Recent studies adapted machine learning based tools for automatic detection of group behaviors in *Drosophila*, facilitating our understanding of collective behavior [29](https://paperpile.com/c/cMiCci/2cq11) and social networks (See **Table 2**; [14,16,24,29,50,51](https://paperpile.com/c/cMiCci/Iv07I+mMg6q+2cq11+xFww6+FVWl2+TeKjH)). For example, it was shown that the internal dynamics in same-sex fly groups depends both on the genetic heterogeneity of the group [16](https://paperpile.com/c/cMiCci/mMg6q) and on previous social experience [24](https://paperpile.com/c/cMiCci/Iv07I). Both supervised and unsupervised learning methods have been developed for the automatic quantification of mating and fighting in *Drosophila* [52–54](https://paperpile.com/c/cMiCci/OfSjU+W64TZ+rIC7h).

**Table 2. Recent studies that have used machine learning tools for the quantification of group interactions in *Drosophila melanogaster***

| No. of individuals | Duration | Mixed sex | Food patch | Body parts tracked? | Year | Reference |
| --- | --- | --- | --- | --- | --- | --- |
| 12 | 30 min | No | No | No | 2012 | [16](https://paperpile.com/c/cMiCci/mMg6q) |
| Up to 24 | 2–5 min | No | No\* | No | 2015 | [29](https://paperpile.com/c/cMiCci/2cq11) |
| Up to 100 | Various | Yes | No | No | 2019 | [50](https://paperpile.com/c/cMiCci/xFww6) |
| 7, 16 | 3-5 h | Yes | No | No | 2020 | [51](https://paperpile.com/c/cMiCci/FVWl2) |
| 10 | 15 min | No | No | No | 2021 | [24](https://paperpile.com/c/cMiCci/Iv07I) |
| **16** | **4 h** | **Yes** | **Yes** | **Yes** | **This proposal** | |

**\*** No food, except for the measuring of aggregation density.

Given the tracking of individual animals and the detection of specific behaviors, studying group dynamics and individual decisions will be done using three approaches: (1) by using predictive models (e.g., [10,55](https://paperpile.com/c/cMiCci/awLRu+5lGdl)), (2) by quantifying network properties [16,56,57](https://paperpile.com/c/cMiCci/M7dvo+bAsvF+mMg6q), including *Clustering coefficient*, *Assortativity*, *Betweenness centrality* and *Global efficiency*, (3) by using a layered social network analysis [58](https://paperpile.com/c/cMiCci/khId), as was employed in behavioral studies (e.g., [59](https://paperpile.com/c/cMiCci/6PTe)). For example, different networks could be extracted for a given dataset, based on mating behaviors alone, aggressive behaviors alone, or on general activity, and the interaction between the networks could be quantified.

**1.3 Central control of social decisions in males and females**Mating behavior in *Drosophila melanogaster* has been the subject of intense research for over a century [60](https://paperpile.com/c/cMiCci/8mdoA). Mating behavior in *D. melanogaster* relies on multiple modalities, including visual, olfactory, auditory and gustatory [6](https://paperpile.com/c/cMiCci/qjU7N). During courtship, males and females display their qualities while analyzing the value of a potential mate. The male initiates courtship and the female decides whether she wishes to mate or not [47](https://paperpile.com/c/cMiCci/KQXli). Upon encountering a potential courtship partner, based on visual and chemosensory cues, the male taps the female’s abdomen to assess her desirability [61,62](https://paperpile.com/c/cMiCci/bkCNU+G0A94). The male then follows the female, extending a wing and vibrating it to generate the courtship song [32](https://paperpile.com/c/cMiCci/P8Oog) and licking the female before attempting to copulate [61,63](https://paperpile.com/c/cMiCci/bkCNU+hItra). Virgin females slow down and open their vagital plate in response to courtship song [31,64](https://paperpile.com/c/cMiCci/9QhrE+UFACz), and ultimately allow copulation. The female’s mating behavior is dependent on her sexual maturity [65](https://paperpile.com/c/cMiCci/2EX5) and on previous mating events, through the immediate effect of a mating plug [66](https://paperpile.com/c/cMiCci/Oav8o) and a slower effect of a sex-peptide, injected by the male during copulation [67](https://paperpile.com/c/cMiCci/3P7M2). Mated females respond to male’s courtship song by accelerating in response to song [10](https://paperpile.com/c/cMiCci/awLRu), extruding her ovipositor [35](https://paperpile.com/c/cMiCci/nx6Vl) and by performing a range of rejection behaviors that include decamping (running, jumping, or flying away), wing flicking, and shoving/fencing [47,48,68](https://paperpile.com/c/cMiCci/KQXli+9aVsW+vFViW).  
Aggressive behaviors of flies have been studied mainly in the context of fighting over food resources, and it has been shown that while some aggressive phenotypes are sex-specific, others are not [39,69,70](https://paperpile.com/c/cMiCci/9ef2n+S6RSu+BgU7R). For example, while hierarchical relationships were seen to form between losing and winning males, hierarchy was not observed in females [39](https://paperpile.com/c/cMiCci/9ef2n). Aggressive behaviors were also documented in the context of mating: males fight with other males when competing over a female [60](https://paperpile.com/c/cMiCci/8mdoA), while an unreceptive female shows aggressive rejection behaviors toward courting male [47,48](https://paperpile.com/c/cMiCci/KQXli+9aVsW). The neural basis of aggressive behavior has been the target of intense research in males [40,71,72](https://paperpile.com/c/cMiCci/w7TOO+nrQPW+s0bOw) and recently also in females [9,42,73](https://paperpile.com/c/cMiCci/TIxLC+UC9fM+bLfXM). As in the case of mating behavior, laboratory studies of aggressive behavior have tended to focus on single, isolated pairs of flies.

Much of the progress in understanding of the neural basis of social behaviors in *Drosophila* is due to the fact that many of the cells who participate in the control of social behaviors, from sensory integration to motors control, express the sex determination genes *doublesex* (dsx) and *fruitless* (fru) [27,74,75](https://paperpile.com/c/cMiCci/2InTk+5HqU+TIGWI). In particular, the *doublesex* (dsx) expressing pC1 neurons (including the fru-expressing male only ‘P1’ subset [74](https://paperpile.com/c/cMiCci/2InTk)) regulate multiple aspects of mating and aggressive behaviors in both sexes [9,76,77](https://paperpile.com/c/cMiCci/WPo5u+TIxLC+JeVX4). pC1 neurons in both sexes have persistent effects on mating and aggression [9,40,76,78,79](https://paperpile.com/c/cMiCci/tGIUS+nhzLL+TIxLC+w7TOO+WPo5u), meaning that their activation effects fly behavior minutes after stimulation offset. Different pC1 subsets are involved in driving persistent mating and aggressive behaviors [9,31,40,42,80](https://paperpile.com/c/cMiCci/UFACz+w7TOO+fVrma+TIxLC+bLfXM), at least in part, by driving an arousal state and by gating the transformation from sensory inputs to mating and aggressive behaviors [9,42,81](https://paperpile.com/c/cMiCci/9L7AJ+TIxLC+bLfXM). pC1 neurons were also shown to regulate the interactions between sex and other behaviors such as sleeping and feeding [82,83](https://paperpile.com/c/cMiCci/oQqr+ZmIuW), possibly driving different behaviors in a threshold dependent manner [84](https://paperpile.com/c/cMiCci/an7ga).

How pC1 activation modulates social decisions in complex environments remains open. For example, when two males court a single female, will the activation of pC1 subsets drive more male-male aggression or more male-female courtship? Will pC1 activation modulate group dynamics or the probability of target switching? Is aggressiveness of females towards a mating pair [44](https://paperpile.com/c/cMiCci/WmGHl) modulated by the activity of pC1 cells?  
[‘pC1’ neurons are heterogeneous groups of sexually dimorphic cells in the male and the female brains, that were previously shown to drive persistent internal states of mating and aggression in both sexes [8,9,31,40,76,80](https://paperpile.com/c/cMiCci/w7TOO+fVrma+WPo5u+dGlb1+UFACz+TIxLC). It has been shown that different pC1 subgroups drive mating and aggressive behaviors, that the effect of pC1 activation persists for minutes beyond the activation period and that pC1 activation also modulates non-social behaviors such as sleeping and feeding.]

1. **Research Objectives**

This project will quantify how social decisions are made by males and females, based on sensory cues, group dynamics and past social experience.

Utilizing advanced computational tools to monitor and quantify social behavior and genetic tools available in *Drosophila melanogaster*, this project has two specific objectives (see **Table 3** - timeline):

1. Develop a novel framework for studying how complex social decisions are made in naturalistic conditions, and how these decisions are modulated by a critical factor: fly density and the level of competition over a sexual partner.
2. Examine how sexually dimorphic central neurons impact social decisions in males and females in a naturalistic environment.

We will test the following concrete hypotheses:

1. An increase in male-to-female ratio will lead to higher aggression between males, more persistent courtship, reduced target switching by courting males, an increase in the probability of females rejecting a courting male (more ‘choosy’ females), longer copulation duration and higher female remating rate.
2. Subsets of the sexually-dimorphic ‘pC1’ neurons bias social decisions in both sexes. In males: courtship persistence and the probability of switching a female target and the ratio of time spent courting a female versus communicating with a competing male. In females, the acceptance rate - in both virgin and recently mated females as well as the response to visual cues (the presence of flies in the field of view), auditory cues (change in speed and turning in response to male song) and tactile.

The first hypothesis draws from theories of sexual selection and previous studies on flies, while the second is based on previous experiments on male-female and male-male dyads. We will combine hypothesis-driven and exploratory approaches: Hypothesis-driven approaches for making rapid progress; exploratory approaches for obtaining a comprehensive description of social decisions in naturalistic social environments and for unexpected findings and novel hypotheses. Experiments will inform and constrain models, and theory will be used to interpret data and refine hypotheses.

This research will expand our understanding of how social decisions are made in more natural scenarios. The computational pipeline used in the study will be made readily available and user-friendly to the wider community as was previously done [85](https://paperpile.com/c/cMiCci/tUBcY). The same tools and methods could be applied for asking followup questions in flies, as well as in other model organisms.

**Table 3** project timeline

missing

***Aim 1. Determine how sensory cues, social context and recent social experience contribute to social decisions in naturalistic social environments***

We will quantify how social decisions in males and females depend on the immediate sensory environment, group dynamics and recent experience of the deciding fly. We will do it in sex-mixed environments, manipulating the fly density and male-female ratio. Social decisions include both categorical and non-categorial ones. Examples for categorical decisions are the male’s choice to initiate or terminate courtship, or his choices to switch between song types, and the females acceptance or rejection of a courtship attempt. Examples of continuous decisions (or responses) are the males song amplitude and the females change-in-speed in response to male song. Some of these decisions were already modeled successfully before, including the choice of song type and amplitude by a singing male. We will extend it to the case of a complex social environment, and test how the existence of other flies impact these decisions. Other decisions were not rigorously described to date, including the decision of a male to switch from courting a specific female to courting another female who crossed the male’s field of view (as we often see in our preliminary data).

We will use two experimental settings. In one we use videography and sound recording, 8 flies and 60 minutes/session; in the other we will record 16 flies for 4 hours, on a food patch, using high speed videography. We will develop a pipeline for automatic quantification of social behaviors (**Table 1**). We will quantify how male and female decisions depend on the degree of competition (varying the male-to-female ratio). The four hour experiment will also allow the quantification of remating, and of history-dependent choices in both sexes. A successful completion of Aim 1 will be an automated pipeline for collecting, analyzing and creating a quantitative description of social decisions in naturalistic environment as a function of (1) ongoing sensory (visual, auditory and tactile) inputs in egocentric coordinates (projected on the fly retinas, aristas and body), (2) sensory-grounded social interaction network features, and (3) previous experience (e.g., recent mating, fighting or rejection).

***Aim 2. Reveal the role of sexually dimorphic central neurons in controlling social decisions in complex environments in males and females***

We will determine the role of known central neurons in the male and female brains in biasing social decisions in naturalistic social environments. This aim too, has hypothesis based and exploratory aspects. We will test the idea that the activity of specific subsets of the sexually dimorphic ‘pC1’ neurons affect the male and female decisions during courtship. We will characterize how the models developed in Aim 1 are modified by the neural activation of pC1 subgroups in both sexes.

Specifically, we aim to reveal how tonic activation (in ‘open-loop’ experiments) and phasic activation (in ‘closed loop’, depending on specific behavioral measures) impact the social decision described in aim 1. For example, we aim to determine if the activation of specific pC1 subsets biases males to persistently court one target female versus switching to another female target, and to measure how pC1 activation impacts the probability of copulation acceptance in mated females (remating). Additionally, if we find that previous social encounters (such as recent rejection of fight in males or the experience of being courted in the case of the female) have predictive power in modeling social decisions, then we will test the effect of pC1 activation on these predictions.

A successful completion of Aim 2 will be a quantitative description of how pC1 activation modulates social decisions in naturalistic environments, using a model which extends the one developed in Aim 1 to include pC1 activation as another feature.

**Expected Significance**

[still missing. Needed?]

**3. Experimental design and methods**

The proposed behavioral studies will be conducted at the University of Haifa. Tracking and pose estimation will be carried out using computational tools that will be developed at the Salk Institute. The two research groups will jointly design the experiments, coordinate the data analysis, and work collaboratively to implement state-of-the-art tools for analyzing behavioral data at multiple spatial and temporal scales. This close cooperation will be achieved via tool and data sharing and personal meetings, both online and through in-person student exchanges. **Critically, neither the experimental group at the University of Haifa nor the computational group at the Salk Institute is in a position to complete the project on its own.**

***3.1 Rationale and general design***

We will develop a computational pipeline for quantifying social decision making in a socially complex and enriched environment, and will quantify how social decisions are modulated by social context (**Aim 1**) and neural modulations (**Aim 2**). As detailed below, our first technical challenges will be to keep track of each one of the 16 individual flies for the extended duration of the experiment (four hours) and to train classifiers for automated detection of multiple social behaviors (**Table 2**). Then, we will use existing frameworks for predicting these behaviors (onset and offset, when relevant) based on egocentric projection of the visual and auditory information (**Fig. 2**), group dynamics and previous events. By the end of Aim 1, we will have a full pipeline for data collection and analysis of social decisions in a complex environment, and a quantitative description of how social context (male:female ratio) modulated these decisions.

The novel pipeline and our behavioral observations will be used as a basis for future studies. For example, focusing on a specific observation in simplified setups, measuring the role of individual senses in driving specific decision (e.g., vision, olfaction and specific odors or pheromones), and manipulating other factors such as fly strain and age, the food substrate and nutritional state.

We choose to focus on quantifying the effect of specific neuronal manipulations on group dynamics and social decisions, aiming to extend our understanding of the neural basis of social communication in more naturalistic settings. Therefore, after establishing a “baseline” description of the wild-type behaviors, in Aim 2 we will use optogenetic manipulations to activate, in a transient manner, specific groups of sexually dimorphic cells in either the male or the female flies.

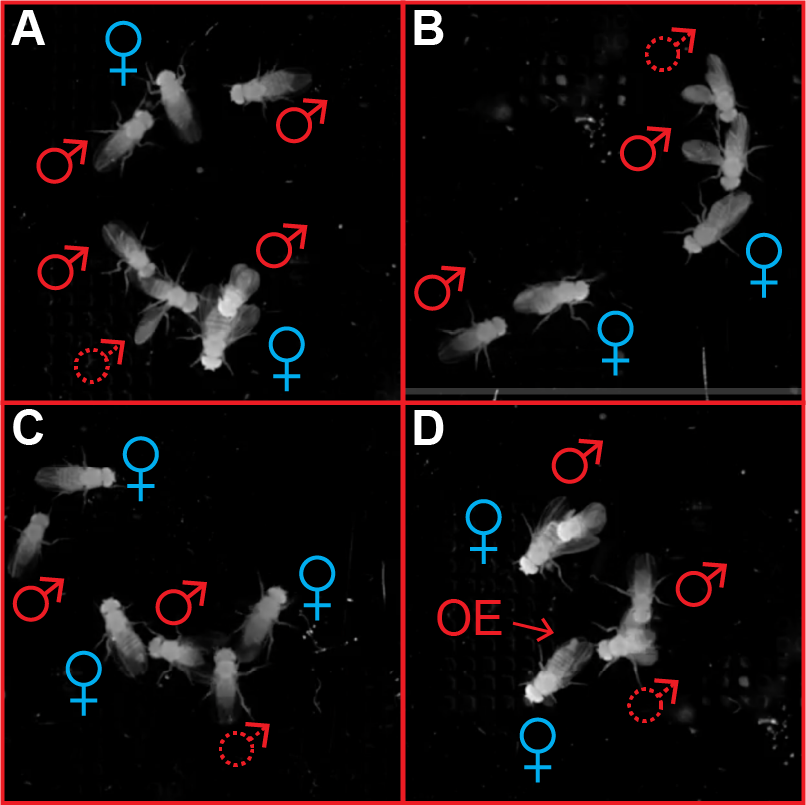
**3.2 Experimental procedures**

**Aim 1: Determine how sensory cues and social context contribute to social decisions in a naturalistic social environments**

***Aim 1.1 - Building and testing behavioral monitoring setup***

We will build a novel setup for collecting data from multiple male and female flies, for an extended duration. The base of the behavioral arena will be covered with fly food. A food substrate was chosen to mimic the natural habitat, and based on previous observations that the presence of food enhances social interactions [70](https://paperpile.com/c/cMiCci/BgU7R). We will build a circular arena with sloped walls to minimize side walking [86](https://paperpile.com/c/cMiCci/Qkfna), keeping 3 millimeters between the food-surface and the ceiling top as previously done [24,52,86](https://paperpile.com/c/cMiCci/Iv07I+Qkfna+OfSjU). To further minimize the occurrences of flies walking on the walls and ceiling we will also use Sigmacoate [10,85](https://paperpile.com/c/cMiCci/awLRu+tUBcY).   
Under laboratory conditions, flies were shown to aggregate on food at densities of ~1-2 flies/cm2 [29](https://paperpile.com/c/cMiCci/2cq11). We will test fly density in pilot experiments. With a density of 1 fly/cm2  and 16 flies we need a diameter of ~4.5 cm for a circular arena. We previously observed that a resolution of 30 pixels/mm is the minimum sufficient for leg tracking [46](https://paperpile.com/c/cMiCci/CDwWB). To also allow fine tracking of subtler events such as arista motion, vaginal plate opening and ovipositor extrusion, tapping and licking, as well as to reduce the probability of identity flips, we will acquire videos at 100 pixels/mm. We therefore choose a camera with a resolution of 5120 x 5120 pixels. We will use a far-IR sensor and filter to avoid data loss during optogenetic activation (**Aim 2**) and since an IR sensor is less sensitive to reflections of ambient room light. Given all these requirements, we will use the Ximea camera model CB262RG-GP-X8G3 and a Gpixel GMAX0505RF Red Fox CMOS sensor with a quantum efficiency (QExFF) of 30% at 850nm.

We will use real-time, hardware-accelerated video compression (~100×) of the high resolution and high frame rate videos. With 8-bits per pixel and 100 fps this would result in ~350GB of compressed data for a 4 hour experiment.  
***Aim 1.2 - Collecting behavioral data***

Flies will be isolated individually to minimize social experience prior to the experiment. Four day old males and females will be inserted to the behavioral arena 60 minutes after the incubator lights switch on to optimize for peak in fly activity. Flies will be individually loaded into the experimental chamber using a custom-built aspirator. We will collect two datasets each day, and will clean the arena thoroughly between experiments. We will collect 16 videos (64 hours) per dataset. Keeping the total number of flies at 16, we will vary the male female ratio: M:F = 8:8, 10:6, 12:4, representing low, medium and high levels of male competition over each female. A 4 hour experiment was chosen to allow the quantification of remaining dynamics.

**Figure 1** Examples of behaviors who involve more than two flies. **A.** male (marked by a dotted circle) is circling around and singing to a copulating pair. **B.** A male (marked by a dotted circle) singing behind a competitor male. **C.** A male switching from competing over one female to courting a different female (to his right). **D.** female extruding her ovipositor while being courted by a male (marked by dotted circle) who is being chased and contacted by another male.

***Aim 1.3 tracking and proofreading pose estimation of multiple flies***

Our preliminary data, collected in an existing setup, suggests that using SLEAP [85](https://paperpile.com/c/cMiCci/tUBcY), we are able to estimate the detailed pose of multiple individuals for an extended period, and to project the audio-visual scene from the point of view of each individual fly (**Fig. 2**).

Currently, the expected rate of identity flips in isolated male-female pairs is 4.9 flips/hour [85](https://paperpile.com/c/cMiCci/tUBcY) . Our preliminary data suggests that in a group of 8 flies (4 males, 4 females) identity flips are still rare. Despite this good performance, in the more challenging setting involving larger groups of flies (16) recorded over longer sessions (4 h), we expect to encounter exponentially more frequent errors, as the potential for identity swaps increases combinatorially with the number of interacting animals and session duration. To address this, we suggest three technological improvements: (1) using a higher spatial resolution (100 pixels per mm, instead of 30 pixels/mm that we are using now), such that each fly is more separable even at close interactions, (2) improve the robustness of SLEAP in identifying identity flips, (3) build a tool for enhanced detection of predicted identity switches.

We will improve the robustness of SLEAP in identifying errors by leveraging state-of-the-art techniques for appearance and trajectory modeling employed for multi-object tracking in the field of computer vision. Specifically, we propose to leverage transformers, a recently developed neural network architecture that employs a mechanism termed “attention”, to capture the relationship between complex data streams; Transformers has been found to be extremely successful for modeling sequence structure across data domains [87](https://paperpile.com/c/cMiCci/5iVRg). This approach has recently been shown to outperform all existing methods for multiple object tracking [88](https://paperpile.com/c/cMiCci/9uahk), and we propose to adapt this approach to capture the relationship between the relative positioning of body parts, static and dynamic appearance features, and postural trajectories in *Drosophila melanogaster*. To achieve this, we will represent each of these features and their relationships via a spatiotemporal graphical representation that will serve as an input to a deep neural network trained to associate animal poses across frames. Unlike all previously described methods for multi-animal tracking, this approach is ideally suited for our project as it enables context integration across multiple modalities of information (visual appearance, pose, dynamics) to achieve highly robust tracking, even in the presence of challenging occlusions that occur during complex multi-individual social interactions.

Even with improved tracking resolution and automated identity tracking, proofreading is still essential for ensuring zero identity flips, however. Proofreading involves jumping between blocks of frames, where the jump-size depends on whether and how closely the flies interact. Our experience is that proofreading takes 10 minutes of proofreading per 30 minute movie. Keeping the identity of each fly in a group of 16 while proofreading is challenging. To overcome this challenge, we are building a tool for enhanced detection of predicted identity switches. In short, we will augment the SLEAP graphical user interface (GUI) with the capability to jump to predicted instances of identity switches and mark, in the relevant frames, the specific flies for which identity flip is most probable. To do this, we will leverage a sizable existing dataset of 11.7 million frames [85](https://paperpile.com/c/cMiCci/tUBcY) that has identity switches manually proofread to train a deep neural network classifier to predict whether a switch has occurred. With this tool, we expect the time it takes to manually proofread a movie to scale with the number of interacting individuals. With 8 males and 8 females we estimate 10 minutes × 4 for 30 minutes of data collection (160 minutes of proofreading for 4hr experiment). We believe that this is an upper limit as (1) we observe in our preliminary dataset, in a given moment, a small number of pairs are actually interacting, and we expect it to happen even less for a long experiment following previous mating events, and (2) in our preliminary movies we did not observe more identity flips in triplets (two males courting a single female) compared to pairs, as typically one pair of flies (male-female or male-male) are in close contact. With the enhanced switch prediction tool we believe that 1 hour of proofreading of 4h movies (8 males, 8 female) is a reasonable estimate. Should this approach fail, we may additionally leverage crowdsourced annotation services. The Deutsch lab is collaborating with the Pereira lab, who is expert in the field of pose estimation and Dr. Perira developed ‘SLEAP’ - the tool that we will be using for pose estimation in the proposed project. A collaboration letter is added to this application.

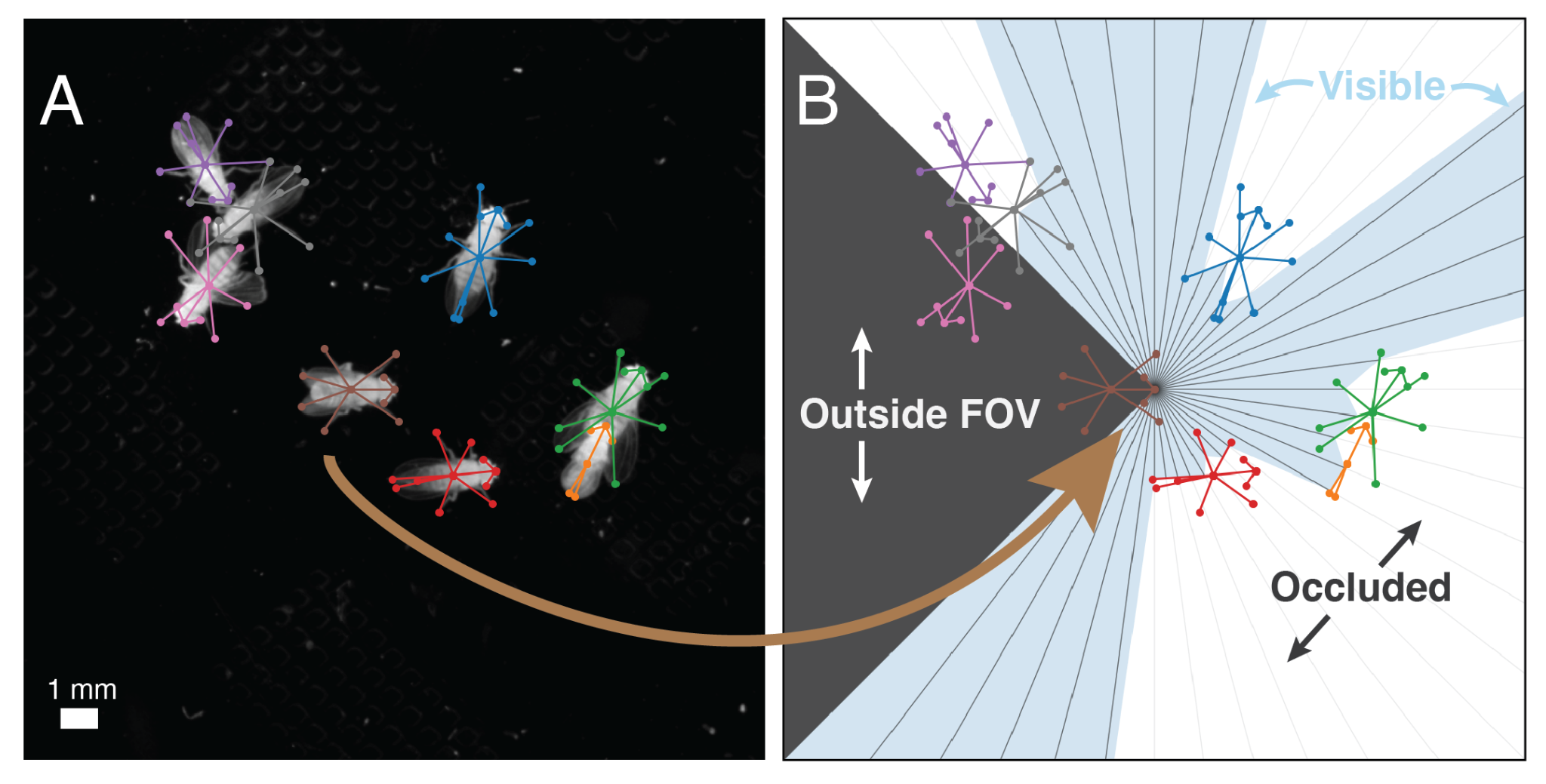
Processing will be done on both campuses on existing GPU clusters. Will make pose tracks available in NeurodataWithoutBorders (NWB) format through the DANDI repository as the SLEAP team has worked with NWB to ensure compatibility. Trained models and labeled data will be made available on OSF with publications. All analysis code will be made publicly available through GitHub throughout development.

**Aim 1.4 - Behavioral analysis**

After behavioral videos are pose tracked, our next step will be to link social decisions with ongoing sensory inputs, past events and group dynamics. We aim to reveal how sensory cues and local group dynamics trigger a male to switch a female target, how the history of previous interactions modulate the female rejecting behaviors. Towards this aim, we need to detect relevant behaviors, quantify group dynamics, and project the sensory input (specifically - visual and auditory) from the point of view of each interacting fly.

(i) **Detecting behaviors** - We will use supervised techniques to automatically extract behaviors (see **Table 1**) at the level of single flies (e.g., singing and ovipositor extrusion [67,89](https://paperpile.com/c/cMiCci/3P7M2+fV5Dl)), pairs (e.g., chasing, tapping or shoving [9,78](https://paperpile.com/c/cMiCci/TIxLC+tGIUS)) and triplets (e.g., partner switching and aggressive interactions between males who are competing over a female). Some of these behaviors were already detected automatically by us and others [9](https://paperpile.com/c/cMiCci/TIxLC) using available techniques for supervised classification [52,90,91](https://paperpile.com/c/cMiCci/OfSjU+ixm4+CC4p). Looking at the preliminary set of data we already collected, we also observed other behaviors that were not reported by the time our preliminary data was collected, including both males and females interacting with a copulating pair (female aggressiveness towards a copulating pair was recently reported by [44](https://paperpile.com/c/cMiCci/WmGHl)). (ii) **Group dynamics** - a few measures were used in the past to quantify group dynamics, based on network theory. These include *Clustering coefficient*, *Assortativity*, *Betweeness centrality* and *Global efficiency* and others [57,92](https://paperpile.com/c/cMiCci/r89yi+bAsvF)*.* Previous studies demonstrated that these group dynamics in *Drosophila* are non-random, and depend both on fly genotype and previous experience [16,24](https://paperpile.com/c/cMiCci/mMg6q+Iv07I). However, how these group dynamics influence moment- by-moment social decisions of individual flies has not been quantified to date. (iii) **Sensory inputs** - based on fine tracking of body, head and arista angles, we will infer the audio-visual scene, from the point of view of each individual fly (**Fig. 2**). The detection of song type depends on the relative angle between the flies [93](https://paperpile.com/c/cMiCci/c5Kgz). Inferring song type from the video alone is possible , though less accurate compared to audio recording by pressure detectors (e.g. [10,94](https://paperpile.com/c/cMiCci/Phzh1+awLRu)). We decided not to use microphones for a couple of reasons. First, as having both microphones and food under the fly, while still detecting high-quality sound is challenging, and second, in order to simplify the data analysis (e.g., assigning sound to each individual). Adding microphones are planned for future experiments.

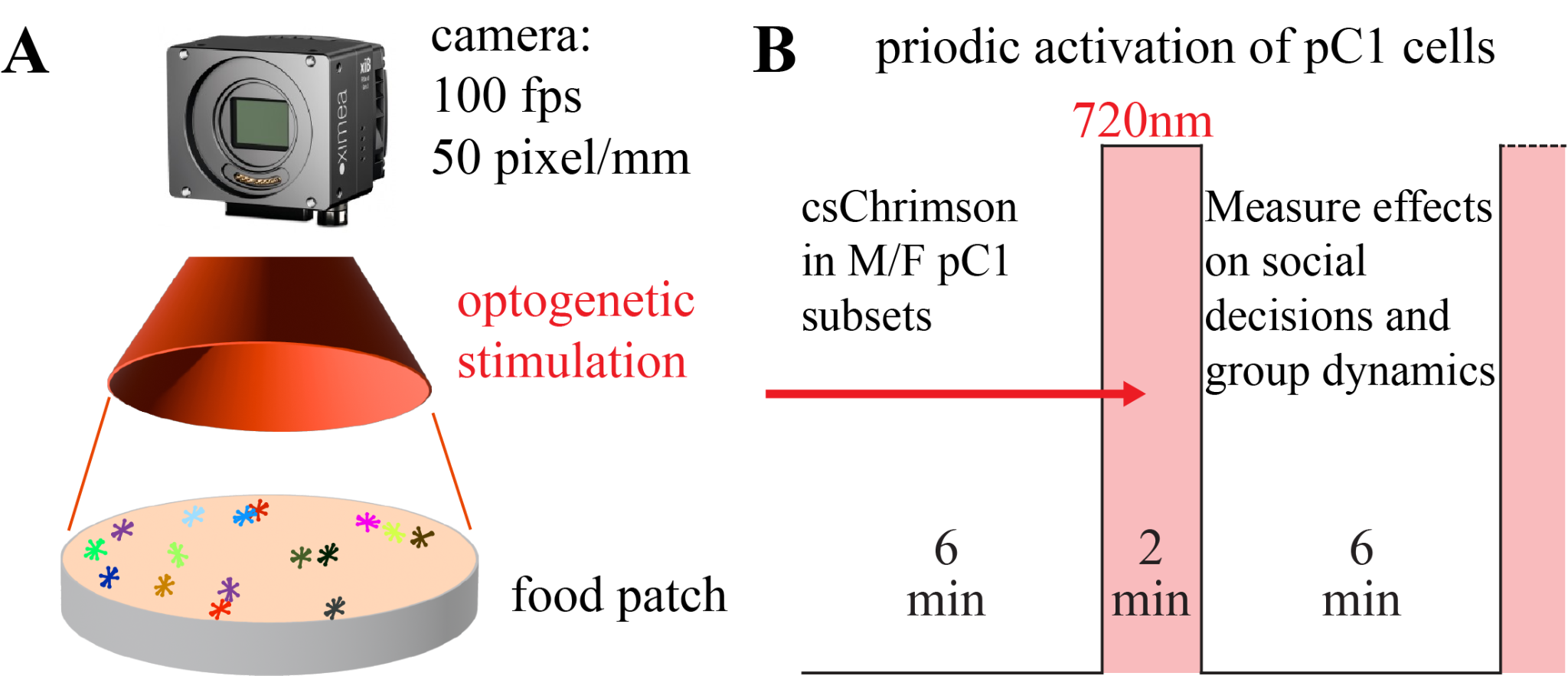
**Aim 1.5 - Predicting behaviors based on sensory cues, local group dynamics and previous experience**

Based on these measures, we will quantify how social decisions are made by individual flies based on ongoing sensory cues, local group dynamics (over some time window) and previous experience. First, we will project the visual and auditory signals to the flies’ egocentric views. Then, we will use an existing framework for the integration of sensory information (in egocentric view) and local group dynamics (as measured. e.g., by *Clustering coefficient*, *Assortativity* and *Betweeness centrality*) with hidden internal state (combining hidden markov models - HMMs and generalized linear models - GLMs - as previously done [55](https://paperpile.com/c/cMiCci/5lGdl)) to predict decisions to initiate, terminate, and switch between behaviors. We will also apply statistical tools for testing if accounting for past events contribute to the power of an HMM-GLM to predict male and female decisions. For example, we will test how visual cues from a courted female and a passing target in the background trigger a male to switch to a different female target, and how a switching decision is modulated by the flie’s recent history (e.g., previous rejections or fights).  
  
**Figure 2** Tracking of individual flies, and projecting to an egocentric view. **A.** Pose tracking multiple files using SLEAP. **B.** Egocentric visualization of central fly with visible regions of field-of-view (FOV) shaded blue.

**Aim 2 - *Reveal the role of sexually dimorphic central neurons in controlling social decisions in complex environments in males and females***

Previous studies suggest that both in males and in females, separate subgroups of the Sexually dimorphic pC1 neurons promote mating and aggression. Critically, activating pC1 neurons in both sexes were shown to drive social phenotypes who last minutes beyond the activation period [9,40,95](https://paperpile.com/c/cMiCci/TIxLC+w7TOO+3dmk9). Here we will test how the activation of pC1 subsets persistently modulates social decisions in complex environments. For example, does the activation of dsx+fru+pC1 cells in males affect the probability of switching a courting target, or the fighting:mating ratio when competing with another male over a female target. Does the activation of dsx+fru-pC1 neurons in males drive a persistent bias towards attacking competitors over courting females? Does the activation of pC1d/e neurons drive females to attack mating pairs [44](https://paperpile.com/c/cMiCci/WmGHl)?

We will activate different pC1 subsets in males or in females (specifically, pC1d/e in females and dsx+fru+pC1, dsx+fru-pC1 in males) periodically: 2 minutes ON, 6 minutes off, 30 times during a 4 hour experiment, using optogenetic activation (**Fig. 3**).

**Figure 3** Schematics of the experimental setup. **A.** flies are tracked using a high-res, hi-speed camera. **B.** Periodic optogenetic stimulation. The 6 minute delay will be used for measuring the decay in the persistent effect of pC1 activation.

The red shifted channelRhodopsin csChrimson will be expressed in the relevant cells in males or in females (but never in both sexes) using available driver lines [9,67](https://paperpile.com/c/cMiCci/3P7M2+TIxLC). A 720nm deep red light will be used for activation. A previous study suggests that while flies have a saddle response to a flash of deep red light (720nm) in dark, having light at the background (as we do in our experimental setup), eliminates this behavioral response. As we will turn the light ON and OFF with no reference to any behavior (meaning, will use an ‘open loop’ protocol), we expect to have different flies engaging in different behaviors during the ON and OFF periods, such that over the course of 30 repetitions, times 16 experiments precondition, we will have enough behavioral events that will allow the comparison between social decisions as a function of time from light-onset and offset. As in aim 1, 850nm light will be used as light for the camera sensors, and a band-pass filter will be used at the camera entrance. The filter profile ensures that the 720 nm light will not be detected by the camera. The behavioral analysis pipeline will be done as in Aim 1.

**3. Potential problems and alternative strategies**

***3.1 Identity tracking.*** As explained in Aim 1, we use a very-high resolution camera (100 pixels/mm) to minimize identity during SLEAP based pose-estimation, and will develop tools for minimizing the manual labor involved in manual proofreading, aimed to ensure zero identity flips after proofreading.

While our tracking of preliminary movies indicates that this approach is likely to prove successful, an alternative approach is suggested: as a last option, we can tag the flies (using an approach similar to [51](https://paperpile.com/c/cMiCci/FVWl2)), but using dots on the back on the fly (could be detected by the IR camera) with 24 options (i.e., 1 or 0 dots in 4 separated areas along the fly back) for identifying 16 flies. A similar approach was used by us and others before, for keeping the identity of the male in male-female dyads [10,96](https://paperpile.com/c/cMiCci/awLRu+V6h0q).

***3.2 Complexity.*** Due to the large space of possible behaviors, it is possible that we will need a larger dataset in order to have enough statistical power. In this case we will collect more data, possibly focusing only on some conditions (e.g., 2 M:F ratios or 1-2 neural manipulations instead of 3). We may also need to modulate the fy number or density. We will start by collecting a preliminary dataset (the one we already collected has less flies, no food, and a lower-resolution camera) before finalizing the experimental plan. The opposite is also possible - if data analysis will turn out to be faster than expected, we will consider testing more manipulations.

**3.3 Optogenetic manipulations.** Comparing the behavior and decisions during and after the activation period may not lead to clear conclusions in some cases, especially if the effect of activation decays over many minutes or since the activation window comes at random times with respect to specific behaviors. There are two alternative approaches we will consider: (1) activating only ½ of the males or female (by feeding only some flies with all-trans-retinal) and comparing the activated vs non-activated ones. (2) using a closed-loop protocol, where we trigger optogenetic activation on specific behavioral instances (as we previously did [85](https://paperpile.com/c/cMiCci/tUBcY)) of specific flies. LED activation of only specific flies has some technical challenges, but will be beneficial for followup experiments. We plan to do it as a second step, by may include within the scope of this project, if needed.

**Closing remarks and outlook**

**== will pave the road**

**==exploratory**

**==Pereira?**

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