THE INFLUENCE OF SENSORY CONDITIONS ON SOCIAL BEHAVIOR AND BRAIN ACTIVITY OF ZEBRAFISH

by

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Disruption in social behavior is characteristic of Autism Spectrum Disorder, a neurodevelopmental disorder that appears in early childhood. Previous experiments in zebrafish showed lesioning of the ventral forebrain reduced social engagement. Results also suggested subjects must be able to see each other to socialize (Stednitz, 2018). Subsequent experiments demonstrated that zebrafish can interact without vision, perhaps using their other senses like the water-pressure mechanosensory and olfactory system. Our study investigates how sensory modalities contribute to social behavior. Measuring behavior in an open field allows for quantification of complex social behaviors like orienting, following, and dispersing. We manipulated sensory modalities by recording behavior in the dark and lateral line ablated conditions. Our results show the loss of the visual input causes a significant 43.3% reduction in orienting behaviors and a 52% reduction in following behavior. When we ablate visual input and mechanosensation, we do not observe a reduction in orienting or following behaviors.

Another outstanding question is which brain regions are activated during social behavior by the contributing senses. We use whole brain immunolabeling with neuronal activity markers as an unbiased approach to identifying and quantifying active brain regions in social and alone conditions. We found the posterior pallium of the forebrain is more active in social than alone conditions. Visual ablated fish had a 44.1% decrease in total telencephalon activity compared to controls. Our study of behavior and corresponding brain activity sheds light on the importance of vision in social behavior and forebrain activity of zebrafish.

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Introduction

Disruption of Social Behavior in Autism Spectrum Disorder

Social behavior is an important part of cognitive and learning processes. Social deficits manifest in Autism Spectrum Disorder (ASD), Schizophrenia, and anxiety disorders. Patients diagnosed with ASD generally display less eye contact, reduced orienting to one's name, and trouble interpreting facial expressions. ASD is also characterized by sensory modulation that impacts resulting cognitive and perception processes. The population of autistic patients continues to grow. In 2018, it was estimated 1 in 59 children[1] are diagnosed with autism. At this moment there is no clear etiology or treatment plan to improve the quality of life for people with autism. Several studies support a multisensory perspective to explain ASD symptoms, where the interaction of multiple sensory modalities is disrupted[2]. A study by Foxe et al, show autistic patients perform similarly in a word matching task in both the audio only and audio-visual condition[3]. However, the subject's performance worsened when background noise during the task increased. The findings further support the idea of impaired multisensory integration as being responsible for reduced communication ability. Basic science research of social behavior in animal models will help us understand how social deficits manifest in different sensory conditions.

Social Behavior in Zebrafish

The zebrafish is an ideal animal model to study social behavior. Zebrafish live in social groups and display shoaling, which is coordinated movement and orientation in

[1] "Autism Facts and Figures."
[2] Baum, Sarah H et al.
[3] Foxe, John J et al.

groups of fish[4]. They also have distinct aggressive and mating behaviors. A study by Dreosti et al (2015) found zebrafish display a preference for other fish starting at three weeks of age[5]. The young zebrafish would spend significantly more time by a divider with other conspecifics on the other side than alone, and demonstrated coordinated movement with the conspecifics behind the divider. Studies have also explored how social behavior impacts decision making and learning processes[6]. Oliveira et al (2015), showed a bystander fish will turn its body to watch a pair of fighting zebrafish and will attend specifically to signaling between the fighting fish.

Orienting, following, and dispersing, are simple behaviors that can make up complicated social interactions. Orienting was defined by Stednitz et al (2018)[7] as a stereotyped turning in a 45 degree angle to visualize a conspecific through a clear partition. In the open field, we still observe zebrafish turning towards one another in this pattern. Following can be defined as parallel swimming, which like orienting, demonstrates the species attraction to others of its kind[8]. Dispersing is defined as the zebrafish swimming away from each other. Though the zebrafish are not facing each other, dispersing may be part of a larger social behavioral motif or the end of a social interaction and is worth measuring. All of these experiments utilizing dyad or partitioned assays point to the visual system as guiding social behavior. However, this study will utilize an open field assay to allow for more complex social behavior beyond orienting. Additonally, there may be other sensory systems involved. For example, kin

[4] *Suriyampola PS.*
[5] Dreosti, Elena, et al
[6] Abril-de-Abreu
[7] Stednitz, 2018
[8] Hinz, Robert C.

recognition which impacts shoaling behavior depends on olfactory and visual cues[9]. This study will interrogate the hypothesis that the visual system drives social behavior and evaluate the contribution of other sensory modalities.

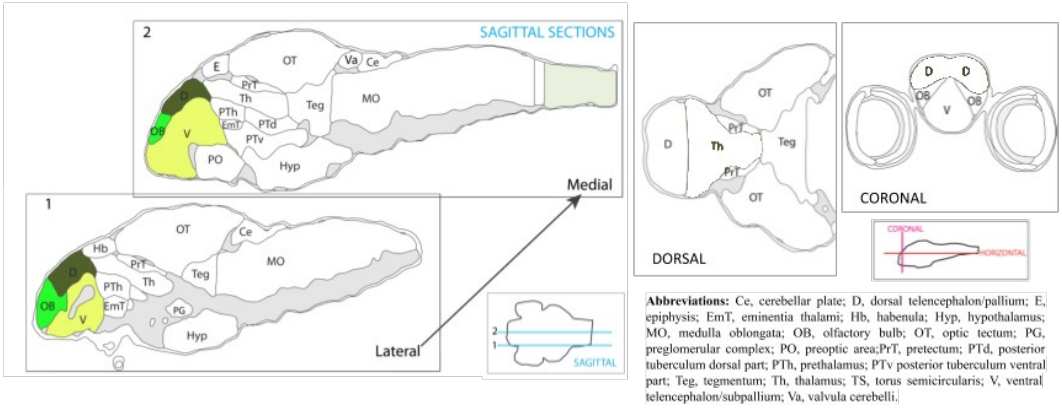
The Zebrafish Forebrain

In addition to characterizing different social behavior patterns, it is important to identify brain regions involved in supporting social behavior. Zebrafish share 70% of their genes with humans and many zebrafish brain regions are evolutionarily conserved with the human brain[10]. The focus of this study will be on the zebrafish telencephalon, which includes the dorsal and ventral telencephalon, also called the subpallium (Figure 1). The telencephalon contains an analog to mammalian caudate nucleus, hippocampus, and amygdala though there is no strong consensus in the literature on how to delineate these regions. Previous studies have pointed to key regions in the zebrafish telencephalon like the ventral telencephalon[11], or the optic tectum[12]. Telencephalic ablation in goldfish produced reduced shoaling behavior with no change in their motor or optomotor response. More specific ablation to the dorsal telencephalon, which researchers believe analogous to the pallial amygdala, had no effect on shoaling. Additionally, researchers tried ablation of the olfactory tract and observed no change in shoaling behavior[13]. Sensory relay has been mapped from the thalamus to the hind brain[14]. We could expect the thalamus to be sensitive to experimental manipulation of sensory systems. At the moment there is little consensus on a particular region within

[9] Hinz, Cornelia., et al
[10] Leung, Louis C., et al.
[11] Stednitz, 2018
[12] Tunbak, 2020
[13] Shinozuka K
[14] Mueller, Thomas.

the forebrain as being important for social behavior. Therefore, we will take whole brain images for unbiased discovery of active regions.

Figure 1: VIBE-Z Segmentation of Zebrafish Brain[15].



Many papers utilize expression of immediate early genes (IEGs) like c-fos and Arc to label active neurons, however these methods have poor temporal resolution and low baseline levels, which makes it difficult to correlate expression with specific behaviors. Antibody labeling[16] of biochemical events are more suited to social behavior experiments. ERK protein (Mitogen activated protein kinase), which belongs to the Ras/ERK pathway[17], is phosphorylated to active pERK during depolarization. While c-fos requires 30 minutes to 2 hours to show signal, pERK appears after 5 minutes[18]. Furthermore, pERK is a better choice because the endogenous levels of ERK protein are detectable to allow for comparison of inactive neurons to active neurons following social behavior.

[15] Ronneberger, Olaf, et al.
[16] See appendix for definition of antibody labeling
[17] Roux, P. P
[18] Randlett, Owen et al.

Zebrafish Sensory Systems

To experimentally manipulate sensory conditions, this study will use pharmacological methods to target the lateral line and olfactory system of zebrafish. The zebrafish lateral line is composed of neuromasts lining the flank of its body[19]. At the center of these neuromasts are hair cells, identical to the ones in the human inner ear. These cells help the organism sense water pressure and current and control balance[20]. The olfactory system in the zebrafish is very similar to human olfaction. Olfaction is important to a wide variety of behaviors necessary for survival, including feeding and mating behavior. Both of these sensory systems can be temporarily damaged by chemicals or injury but the cells will eventually fully regenerate; olfaction in 72 hours[21], lateral line in one week[22].

Specific Aims

1. We will investigate what sensory systems support social behavior. We will test the hypothesis that visual input is important for social behavior. We will measure orienting, following, and dispersing behavior with specific sensory modalities ablated. Social behavior experiments will be performed in an open field assay, which allows for the measurement of more complicated social behaviors and social behavior informed by other senses. We expect the visual system to be primarily important for social behavior, particularly orienting. We expect lateral line to have small effects on the frequency of following and dispersing behavior.

[19] Torregroza, Ingrid, et al..
[20] Gompel, N, et al.
[21] Calvo-Ochoa, Erika, et al.
[22] Mekdara, Prasong J., et al.

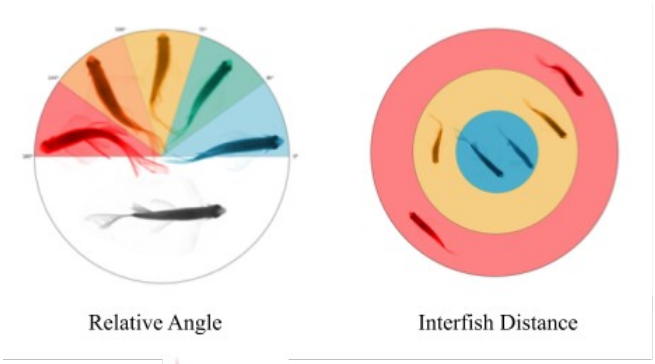
2. We will investigate how social behavior and sensory input affects forebrain activity. We will test the hypothesis that specific regions in the telencephalon are active during social behavior. We will use pERK/ERK ratio to quantify total activity and identify regions important to social behavior. We expect total activity in the telencephalon to be increased in social fish compared to alone fish and that active telencephalon regions will be unique to social fish.

Methods

Behavioral Data Collection

One to three month old wild type (*Danio rerio*) fish were placed in pairs or alone in a 9 inch diameter open field container. Depth of water was kept at 1” to restrict fish ability to dive or jump from the arena. Behavior was tracked for 10 minutes using a Mightex camera (7800 frames total, 20ms exposure) using infrared illumination and a visible light blocking filter. Images were processed by ImageJ script that subtracts the image background and a Python script[23] that tracks moving fish. The script automatically tracks angle and distance of each fish (Figure 2). Behavior paramenters are manually defined[24]. Orienting is defined by angles 144°-216° and interfish distance of one fish length or less. Following is coded as angle 0°-36° and 324°-360° and interfish distance of less than one fish length (figure 2)[25].

[23] See appendix for preferences tracking code
[24] See appendix for behavior coding python code
[25] See appendix for social behavior examples



Motif	Orienting	Following	Dispersing
Angle	<160	<20	>20, <160
Distance	<50	<50	>50

Figure 2: Schematic for Automatic Coding of Behavior

Sensory Manipulation

Ablation of the visual system was achieved by recording behavior in a dark room. Infrared light, undetectable to the subjects, allowed the camera to capture behavior. Manipulation of the olfactory system was achieved by specific killing of the olfactory epithelium with detergent. Subjects were sedated using 5mL of MS-222 placed in 500mL of water, before detergent was applied to each olfactory epithelium[26] . Fish were allowed 20 minutes to recover from the sedation and the procedure before starting the experiment. The mechanosensory system was manipulated by exposing fish to an antibiotic solution (12.5g erythromycin/500mL of water), which kills the hair cells along the lateral line nerve system[27]. Fish were placed in the antibiotic solution for 10 minutes then directly into the arena for experiment.

[26] Iqbal & Byrd-Jacobs (2010)
[27] Harris et al (2003).

Tissue Protocol

Subjects were humanely sacrificed immediately following behavior data collection and their heads were fixed in 4% PFA solution. After 1.5 hours, brains were dissected and left in 4% PFA overnight at room temperature. Brains were cleared using CUBIC protocol[28]. First tissue was placed in CUBIC 1 solution (25 wt% of 80 wt% Quadrol, 25 wt% urea, 15 wt% Triton X-100 in dH_2O) at 37°C for three days. Tissue was treated with a 5% BSA, 2% goat serum in PBST blocking solution overnight and rinsed before antibodies were applied. ERK antibody and pERK antibody were applied overnight with gentle rocking. Whole brains were rinsed several times 0.5% PBSTx the following day. Fluorescent secondary antibodies were applied overnight in darkness with gentle rocking. Tissue was rinsed again in 0.5% PBSTx before mounting with Prolong Gold antifade mounting media (Invitrogen).

Antibody	*Company*	*Catalog #*	*Host*	*Concentration*
ERK	Cell signaling	4696S	Mouse	1:500
pERK	Cell Signaling	4370S	Rabbit	1:500
Anti-mouse (488nm)	Invitrogen	A21141	Goat	1:1000
Anti-rabbit (546nm)	Invitrogen	A11035	Goat	1:1000

Table 1: List of Antibodies used in Immunohistochemistry Tissue Protocol

[28] Marquart, GD.

Confocal Microscopy

Activity labeling with pERK and ERK, a marker of neuronal activity, benefits the integrity of the experiment by allowing for an unbiased investigation into the whole brain of subjects. Tissue was visualized using fluorescence confocal microscopy (Leica DMI8-CS and a 10x objective). Overlay of DAPI, ERK and pERK channels was achieved by Leica software. Scanning proceeded in an order that minimized interference of channels and prevented bleaching of the fluorescent signals in the tissue.[29]Images were realigned automatically using the ERK channel of a reference brain. Quantification of signal achieved by registering images by ERK channel in Fiji using the Computational Morphometry Toolkit (CMTK)[30] (Neurodebian OS, https://www.nitrc.org/projects/cmtk).

Quantification of Fluorescent Intensity

Images were processed with Gaussian blur[31] at 2 microns. The pERK two dimensional projected image was divided by the ERK projected image. Otsu automatic threshold[32] was applied to each image to distinguish background signal from true activity. The Otsu algorithm[33] creates a histogram of each pixel for adaptive image binarization and segmentation of active regions. Otsu automatic thresholding was necessary to control for differences in image acquisition settings.

Statistical Analysis

[29] See appendix for merged images
[30] Rohlfing, T., et al.
[31] Getreuer, Pascal.
[32] Otsu, Nobuyuki.
[33] Dong, Liu, et al.

Statistical analysis[34] was performed in Python (Python Software Foundation, https://www.python.org/). Two Way Anova and Tukey Post Hoc Tests performed using open source Pinguoin[35] on Python. Data visualization[36] was performed on Python using Scipy ecosystem[37] including seaborn.

[34] See appendix for statistical analysis in python
[35] Vallat, R.
[36] See appendix for seaborn visualization code
[37] Waskom, Michael et al.

Results

Result 1: Visual Input Drives Social Behavior

The first aim of this project was to evaluate which sensory systems support social behavior. Visual input and mechanosensation was experimentally ablated (Table 2) and the effect on orienting, dispersing, and following behavior was measured as frequency (total occurrences of specific behavior/all behavior) for each experiment.

Conditions	Visual Input	Mechanosensation
Social control	**yes**	**yes**
Social blind	**no**	**yes**
LLA	**yes**	**no**
LLA blind	**no**	**no**

Table 2: Experimental Groups for Result 1

Coding of behavior by angle and distance allowed each frame of the ten minute experiment to be categorized as orienting, following, or dispersing. We hypothesized that visual input was most important to social behavior. In social control and lateral line ablated (LLA) fish, orienting was the most common social behavior followed by dispersing and following. In visual ablated (blind) and LLA blind fish, dispersing was the most common behavior followed by orienting and following (Figure 2A).

Orienting

Results from Two-Way ANOVA revealed a significant vision effect, $F(1,24)= 51.28$, $p<0.001$, $\mu\text{^}2=0.70$), where there is more orienting in the control than in the dark

(Figure 2B). There was no significant lateral line effect on orienting (Figure 2C), $F(1,24)=0.640$, p=0.432, μ^2=0.026), and no significant interaction between visual and lateral line effects, $F(1,24)=3.35$, p=0.080, μ^2=0.123. Both social control (M=0.534, SD= 0.0825) and LLA (M=0.476, SD= 0.09256) fish had significantly more orienting than both the blind (M= 0.303, SD=0.0419) and LLA blind condition (M=0.342, SD=0.0342) (Figure 2A). When lateral line is present, loss of visual input causes a 43.3%[38] reduction in orienting behavior. Furthermore, there was no significant difference between the social control and LLA (p=0.756, g=0.637), which supports our hypothesis that visual input is primarily important for orienting.

Following

Results from Two-Way ANOVA revealed a significant vision effect, $F(1,24)=9.258$, p=0.005, μ^2=0.242), where there is more following in the control than in the dark (Figure 2B). There was no significant lateral line effect (Figure 2C), $F(1,24)=0.021$, p=0.885, μ^2=0.001), and no interaction between visual and lateral line ablated conditions, $F(1,24)=0.0261$, p=0.613, μ^2=0.009. In Tukey-HSD post hoc test, social control fish (M=0.152, SD=0.0627) had significantly more following behavior than blind (M=0.0734, SD= 0.0223) fish (p=0.002, g=1.58) (Figure 2A), which indicates visual ablation results in a 52% decrease in following. Interestingly, there was no significant increase in following behavior in LLA (M=0.143, SD=0.115) fish compared to LLA blind fish (M=0.0877, SD=0.0225) (p=0.255, g=0.650).

Dispersing

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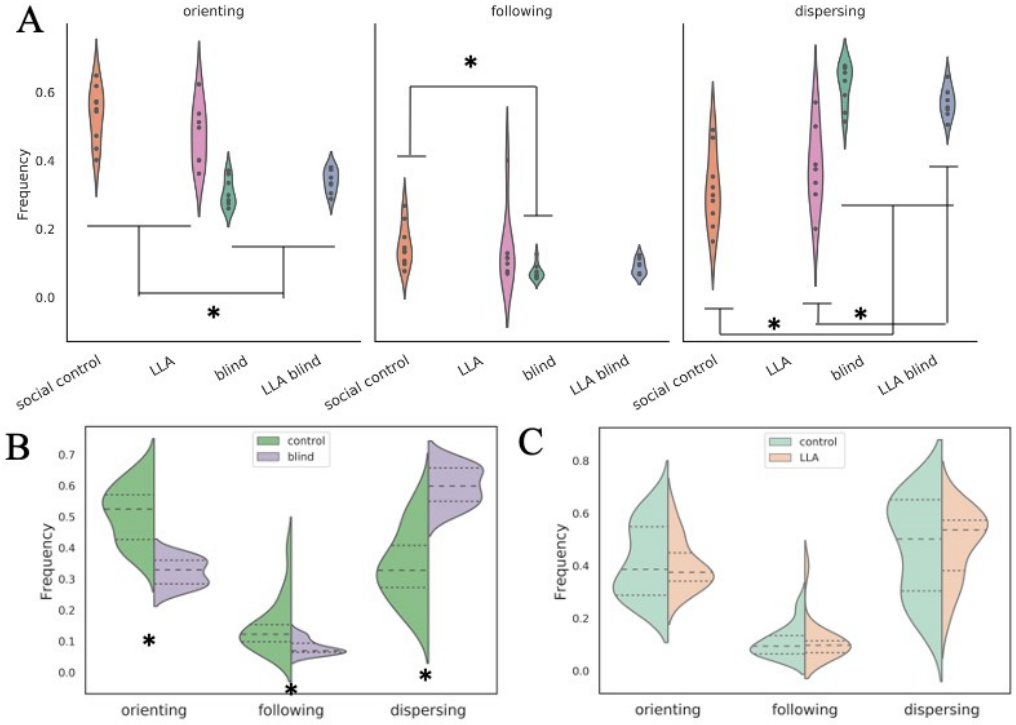
38 $$\frac{(V_2 - V_1)}{|V_1|} \times 100$$

Results from Two-Way Anova revealed a significant vision effect, $F(1,24)= 67.52$, $p<0.001$, $\mu^2=0.700$), where there is more dispersing in the control than in the dark (Figure 2B). There was no significant lateral line effect (Figure 2C), $F(1,24)=0.024$, $p=0.878$, $\mu^2=0.001$), and no interaction between visual and lateral line ablated conditions, $F(1,24)=3.711$, $p=0.064$, $\mu^2=0.113$. Tukey-HSD post hoc test, social control fish (M=0.314, SD=0.109) had significantly less dispersing behavior than both the social blind (M=0.624, SD=0.0615) ($p<0.001$, g=3.33) and LLA blind fish (M=0.571, SD=0.0437) ($p<0.001$, g=2.86). Additionally, LLA (M=0.381, SD= 0.123) fish had significantly less dispersing than LLA blind fish ($p=0.00567$, g=2.00) (Figure 2A). These results suggest loss of visual input increases dispersing behavior and that mechanosensation is unimportant for dispersing behavior.

Figure 3: Social Behavior is Visually Driven



A. Frequency of behavior types in all experimental conditions. Sample sizes for groups: n=9 (social control), n=9 (blind), n=7 (LLA), n=8 (LLA blind).

B. Frequency of social behavior with visual input manipulated. Significant effect of vision on all behavior types. Sample size for groups: n=26 (control), n=17 (blind).

C. Frequency of social behavior with lateral line manipulated. No significant effect of lateral line on behavior types. Sample size for groups: n=18 (control), n=15 (LLA).

* Indicates $p<0.01$

Result 2: Visual Social Activity in the Telencephalon

The second aim of this study was to evaluate how social behavior and sensory input affect forebrain activity and region of activity. We measured brain activity in fish with a conspecific and alone as well as ablated the visual system (Table 3).

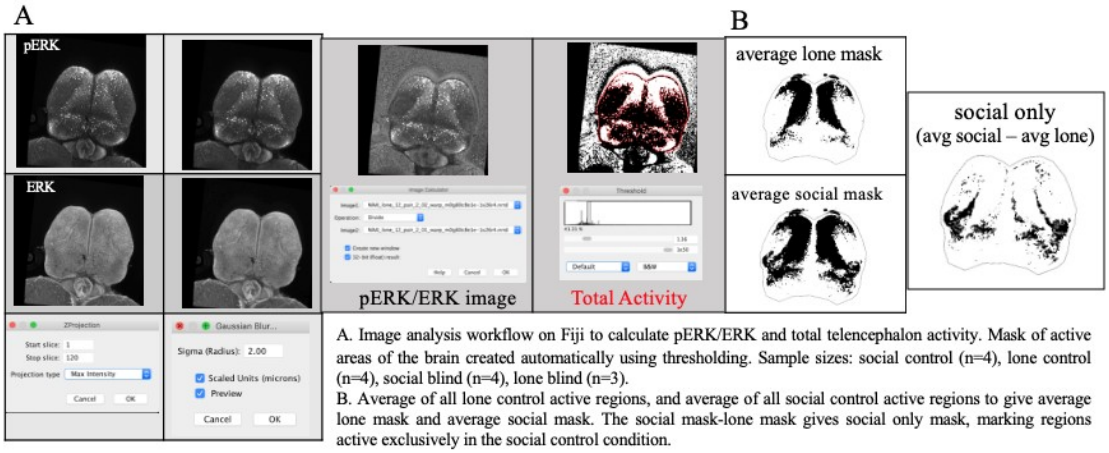
Conditions	Conspecific	Visual Input
Social control	**yes**	**yes**
Social blind	**yes**	**no**
Lone control	**no**	**yes**
Lone blind	**no**	**no**

Table 3: Experimental Groups for Result 2

Brain activity was quantified by the fluorescent intensity of pERK/ERK. Total activity regions for each subject were automatically drawn (figure 3A). We measured the mean intensity, minimum intensity, maximum intensity, standard deviation, and area. To control for differences in the area, I calculated a value of total intensity by multiplying the mean intensity by the area. CMTK Registered brain images from social control and lone control fish were averaged for unbiased selection of average regions of total activity in social and lone conditions (Figure 3B). When I subtract the lone mask from the social mask, I obtain a social-only mask, which covers the areas exclusively active in social conditions. Average social and lone masks appear to cover the medial region

of the telencephalon and the anterior region of the telencephalon. However, areas of the posterior region of the telencephalon, including parts of the thalamus, are social only. From behavioral data, we know visual input supports social behavior. Therefore, we expect greater telencephalon activity in the social control group compared to social blind group. To make the connection between social behavior and the telencephalon, we expect the social groups to have a greater mean intensity compared to lone groups, particularly in the social only region.

Figure 4: Identifying Total Activity and Social Specific Activity in the Telencephalon

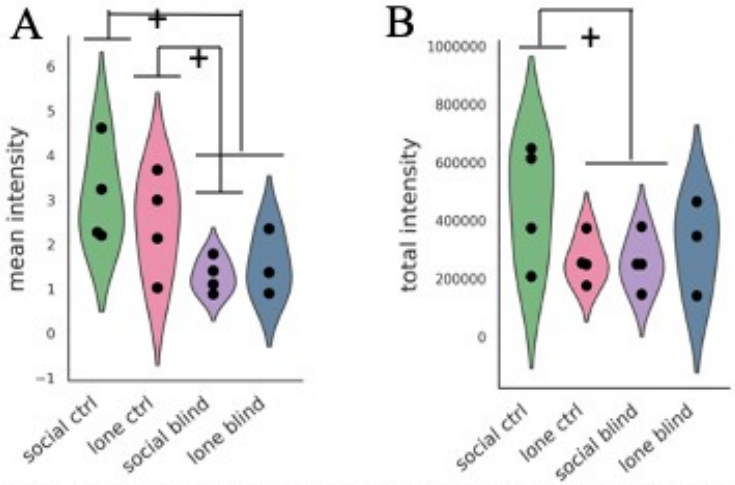


A. Image analysis workflow on Fiji to calculate pERK/ERK and total telencephalon activity. Mask of active areas of the brain created automatically using thresholding. Sample sizes: social control (n=4), lone control (n=4), social blind (n=4), lone blind (n=3).
B. Average of all lone control active regions, and average of all social control active regions to give average lone mask and average social mask. The social mask-lone mask gives social only mask, marking regions active exclusively in the social control condition.

Initial results show higher mean pERK/ERK intensity in the total active areas of the social control (M=3.10, SD=1.10) and lone control (M=2.45, SD=1.14) groups than in the social blind (M=1.29,0.391) and lone blind (M=1.42, SD=0.361) groups (Figure 5A). The significant vision effect in a Two Way ANOVA $F(1,11)=8.453$, p=0.014, μ^2=0.435) seems to suggest vision explains the increased activity. However, when area was taken into account in total intensity, there was no significant vision effect $F(1,11)=1.283$, p=0.283, μ^2=0.104) (Figure 5B). The difference between total intensity of social control (M=458000, SD=186000) and the lone control (M=262000,

SD=81900) was more dramatic (Figure 5B). Additionally, social blind (M=256000, SD=95500) fish had 44.1% decrease in total intensity in the telencephalon compared to social control fish. There was no statistical difference in total intensity between lone control fish and social blind fish (p=0.915) or lone blind groups (M= 470000, SD=179000) (p=0.638). This evidence suggests that the majority of activity in the telencephalon is visual social, that is it depends on both visual input and social behavior, which is only in the social control group.

Figure 5: Social Visual Input Increases Total Activity in the Telencephalon

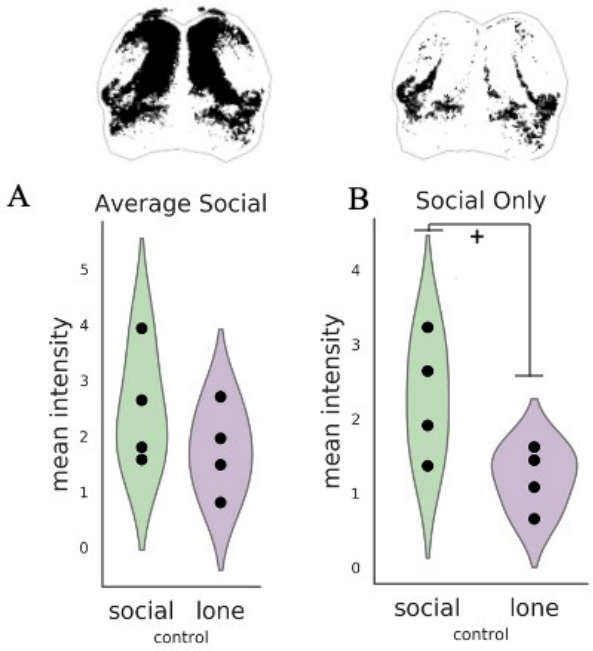


A. Mean intensity in 'total active' areas of telencephalon in each condition. Sample sizes: social control (n=4), lone control (n=4), social blind (n=4), lone blind (n=3). Comparing social control to social blind (p=0.0240) and lone blind (p=0.0812), and lone control to social blind (p=0.103), hedges g>1.00.
B. Total intensity of total active areas in each condition. Large effect when comparing social control to lone control (p=0.127), and social blind (p=0.123) hedges g > 1.0.
+ indicates strong effect size (hedges g>1.00)

Moving forward with the social control group, we can quantify intensity inside the social-only mask and compare to the lone control group. Visual input is intact in both.

The average social region did not show significant differences between social (M=2.49, SD=1.07) and lone (1.74, 0.800) (p=0.304, *g*= 0.691). However, the mean intensity in the social only region was greater in the social control group (M=2.29, SD=0.815) than in the lone control group (M=1.20, SD=0.427) (p=0.056, *g*= 1.50). This result confirms social behavior drives activity in specific regions in the telencephalon, largely the posterior telencephalon. While we have yet to develop a mask of average social dark activity that could be used to compare social only and visual only regions, the social only mask already takes into account visual regions active when a fish is alone.

Figure 6: Social Visual Input Increases Activity in Exclusively Social Regions



A. Mean intensity in averaged social area in social control (n=4) and lone control (n=4). p=0.304, hedges g=0.691
B. Mean intensity in social only area. p=0.056, hedges g=1.5. Approaching statistical significance.
+ indicates strong effect size (hedges g>1.00)

Discussion

Results from this study support the hypothesis that visual input principally drives social behavior. Blind subjects demonstrated approximately 43.3% decrease in orienting and 52% decrease in following behavior compared to controls. Mechanosensation appears unimportant because the lateral line ablation had no effect on frequency of behaviors measured. We can also conclude that telencephalon activity is driven by visual social behavior. Loss of visual input caused a 44.1% decrease in telencephalon activity. There was no difference in total intensity between the lone control, social blind and the lone blind group. We would expect more telencephalon activity in social blind fish compared to lone blind fish, because there is still social behavior occurring. Yet, it appears social behavior does not affect total telencephalon activity when there is no visual input. This could be due to less social behavior overall, as observed in the behavioral data. It could also be other regions,, beyond the telencephalon are involved. Nevertheless we can conclude loss of visual input reduced total telencephalon activity and reduced social behavior. The correlation between telencephalon activity and visual input requires increased sample size and comparison with social blind regions.

This study contributes additional evidence to previous studies that establish the telencephalon as the seat of social behavior. Our results agree with Tunbak's study that report increased forebrain activity in socially reared fish compared to socially isolated and anti-social fish[39]. There was a 74.8% increase in telencephalon total activity in social fish compared to lone fish, and a 90.8% increase in activity in social only regions.

[39] Tunbak, et al.

It appears the socially important region of the telencephalon is primarily located in the posterior region of the telencephalon. This region may include neurons from the ventral telencephalon, pre-thalamus, and analogs of the mammalian hippocampus and amygdala. Rendering the social only mask in three dimensions will elucidate anatomical regions.

Another plausible step is to characterize the neurons in the social only region identified in this study. The ventral telencephalon region that proved important for orienting behavior by Stednitz et al (2018) contained Lhx8 cholinergic neurons. The Lhx8 gene has been shown to be important in the development of cholinergic neurons in the telencephalon in mice, particularly the ventral pallium and hippocampus[40]. Such neurons may also be present in the social-only region. Other types of neurons may also populate this social-only region. Dopaminergic neurons have also been implicated. Socially isolated zebrafish showed decreased shoaling and increased anxiety along with reduced dopamine metabolite levels[41]. Additionally, oxytocin receptors (zOT) have also been shown to be important for social preference.

We are continuing to develop unassisted methods for detecting novel complex social behavior motifs. In this study we used angle and distance as parameters for three behavior types; however, preliminary results from automatic hierarchical clustering of data suggests seven or more unique behavior types.

While more basic research on sensory interaction in social behavior is needed, this study may inform educational interventions that addresses the importance of visual

[40] Zhao, Yangu et al.
[41] Shams, Soaleha et al.

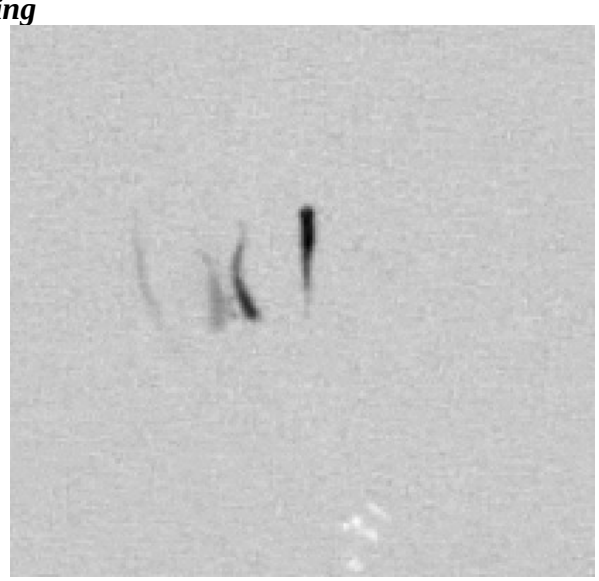
input in normal social behavior. Many occupational therapies work to improve visual tasks like ASD patient's eye contact and recognition of facial expressions. Additional investigation of social behavior activity in the zebrafish telencephalon may support clinical findings that show overactivity and increased volume in the amygdala and the hippocampus[42].

[42] Amaral, David G., et al.

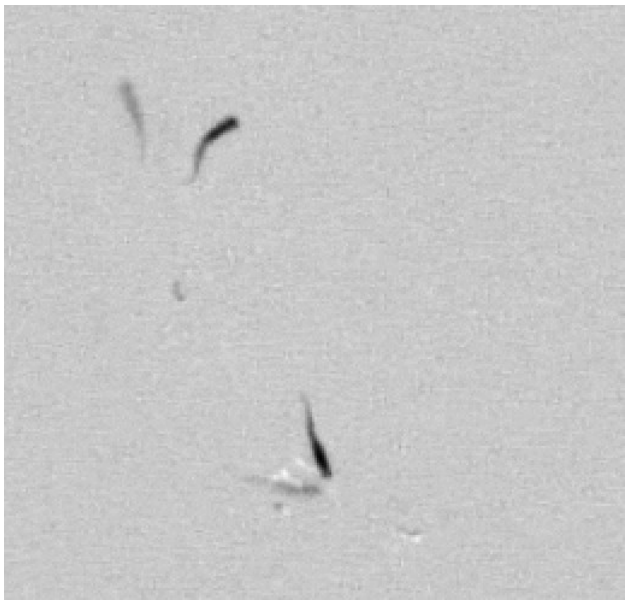
Appendix

Examples of Social Behavior:

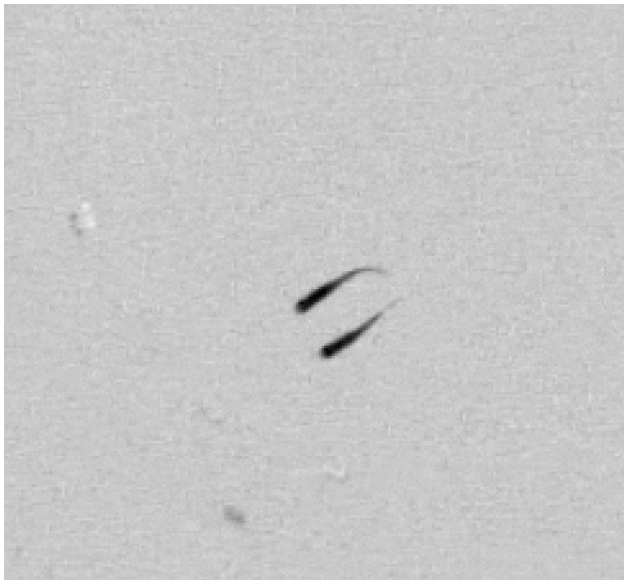
Orienting



Dispersing



Following



Preferences Script Output:

```
import cv2
#import cv2.cv as cv
import numpy as np
from numpy import genfromtxt
import glob
import os
import math
import matplotlib.pyplot as plt

from functions import *
from prefs import *

#### MAIN SCRIPT START
# initialize variables
com = [-1,-1]
angle_part = [np.nan] * 2
kernel = np.ones((5,5), np.uint8)


color_bank = [[107,89,132],[116,135,109],[172,30,164],[241,80,12],
[234,150,0],[18,134,73]]
h,s,v = 0,200,150

for exp_id in folders:

    dirstring = data_dir + "/" + str(exp_id) + "/*" +
img_extension;
    img_list =glob.glob(dirstring); img_list =
natural_sort(img_list)
    minframe = 0; maxframe = len(img_list)
    #print(img_list)
    for roi in range(0,num_rois):
        print(exp_id,roi)
        temp_dir = results_dir + "/"
        create_path(temp_dir); create_path(results_dir + exp_id +
str(roi) + "_images/")
        x_min = roi_bank[roi][0]; y_min = roi_bank[roi][1]; x_max
```

```
= roi_bank[roi][2]; y_max = roi_bank[roi][3]
               frame = cv2.imread(img_list[0]); frame =
process_frame(frame,x_min,y_min,x_max,y_max)#frame = frame[y_min:y_max,
x_min:x_max]; #
               height,width,depth = frame.shape
               trace = np.zeros((height,width,depth), np.uint8); trace =
255-trace
               for frame_number in range(minframe,maxframe):

                   data_pt = []
                   filename = img_list[frame_number]
                   frame = cv2.imread(filename); frame =
process_frame(frame,x_min,y_min,x_max,y_max)#frame = frame[y_min:y_max,
x_min:x_max]; #frame = process_frame(frame)
                   gray = cv2.cvtColor(frame, cv2.COLOR_BGR2GRAY)
                   clahe = cv2.createCLAHE(clipLimit=2.0,
tileGridSize=(8,8))
                   gray = clahe.apply(gray)
                   thresh = cv2.threshold(gray, threshold, 255,
cv2.THRESH_BINARY_INV)[1]
                   thresh = cv2.dilate(thresh, kernel, iterations = 1)
                   new_frame = distance_transform(thresh,2,3); #new_frame
= 255-new_frame
                   #ret, markers =
cv2.cv.connectedComponents(np.uint8(new_frame))
                   #markers = cv2.cv.watershed(new_frame,markers)
                   new_frame = cv2.cvtColor(new_frame,cv2.COLOR_GRAY2BGR)

                   #cv2.imshow('newframe',new_frame)
                   contours,thresh = find_contours(new_frame)

                   ##If you find any contours, do some stuff
                   if len(contours) > 0:
                       ##Calculate center of mass for all contours
                       com_list = center_of_mass(contours)

                       ##Draw all contours
                       for contour_index in range(0,len(contours)):
                           contour = contours[contour_index]
                           ##Find the center of mass of all of the
contours
                           com = com_list[contour_index]
                           area = cv2.contourArea(contour)
                           ##Calculate the tail position and angle of
orientation
                           x,y,w,h = cv2.boundingRect(contour);
dimensions = [x,y,w,h]
                           #cv2.rectangle(frame,(x,y),(x+w,y+h),
(170,170,170),1)
                           distal_pt = tail_detect(thresh,com,dimensions)
                           if distal_pt[0] > 0 and com[0] > 0:
                               distal_pt = tuple(distal_pt)
                               frame,angle =
orientation_detect(frame,contour,com,distal_pt)
                           else: angle = -1
                           data_pt.append(com[0]);
data_pt.append(com[1]); data_pt.append(angle); data_pt.append(area)
                       ##Do some drawing
                           ##Convert to HSV
                           frame = cv2.cvtColor(frame,cv2.COLOR_BGR2HSV)
                           h = int(angle*0.5); #print(h)
                           color = (h,s,v)#(int(angle),s,v)
```

```
                            #color = (color_bank[contour_index]
[2],color_bank[contour_index][1],color_bank[contour_index][0])
                            cv2.drawContours(frame, contours,
contour_index, color, -1)
                            frame = cv2.cvtColor(frame,cv2.COLOR_HSV2BGR)
                            cv2.circle(frame, com, 2, (255, 255, 255), -1)
                            cv2.circle(frame,distal_pt,2,(255,255,255),-1)
                            if frame_number > 1:

                                try:
                                    prev_com =
prev_com_list[contour_index]
                                    cv2.line(trace, (com[0],com[1]),
(prev_com[0],prev_com[1]), color, thickness=1)
                                except: pass
                        prev_com_list = com_list
                        #print(data_pt)
                        #data_str = str(data_pt[0]) + "," +
str(data_pt[1]) + "," + str(data_pt[2]) + "," + str(data_pt[3]) + "," +
str(data_pt[4]) + "," + str(data_pt[5])
                        text_path = temp_dir + exp_id + str(roi) + ".csv"
                        write_text("text",text_path,[data_pt],"%s")

                    cv2.imshow('frame',frame)

                    if save_frames == True:
                        img_dir = temp_dir + exp_id + str(roi) +
"_images/"+str(frame_number) + ".jpg"
                        cv2.imwrite(img_dir, frame)
                    #cv2.imshow('trace',trace)
                    if cv2.waitKey(delay) & 0xFF == ord("q"):
                            break
            #cv2.destroyAllWindows()
```

Behavior Analysis Code

```
      #Written by Sarah Stednitz 2019
      import numpy as np
      import pandas as pd
      from glob import glob


      import seaborn as sns
      #import matplotlib.pyplot as plt
      #import math
      #from scipy.stats import gaussian_kde
      #import scipy
      #print(scipy.__version__)


      ## Change this directory here to the one containing .csv files!
      directory = "/home/jovyan/Bi410/Lateral_Line/"
      filetype = ".csv"
      ## Change the group (light, dark) and it will only analyze those
text files
      ## leave it as "" to do all of them
      group = ""


      #### VARIABLES TO MESS WITH
      ## threshold for how close they have to be to be considered
"following" or "orienting", everything else is dispersing
      dist_thresh = 50
```

```
      ## threshold for how similar an angle has to beto count as
following
      follow_thresh = 20

      ## This is the pattern glob uses to generate files
      pattern = "*" + group +  "*" + filetype
      ## Now make a list of files based on those that match the pattern
in your specified directory
      file_list = glob(directory + pattern)

      ## This makes a list of ids that don't contain the whole file path
(cuts off the directory + file extension part)
      ## Helps for pretty printing later
      id_nums = []
      for i in file_list: id_nums.append(i[len(directory):len(i)-
(len(filetype))])

      ## Double check to see if it successfully detected the files.
      ## If this prints nothing, make sure your directory and patterns
are set correctly!
      print(id_nums)

      ###### Put your functions here!
      ### This part is necessary to turn out pizza plot into a "calzone
plot" - because 359* really is the same as 1*
      ### The maximum difference between any two angles can only be 180*
and this corrects for that
      def calzone(data):
          for i in range(0,len(data)):
              if data[i][1] > 180:
                  data[i][1] = (data[i][1]-180)%360
                  data[i][1] = np.abs(data[i][1] - 180)
          return data

      ### MAIN SCRIPT!
      ## Loop through all of the ID numbers
      for id_num in id_nums:
      ## Create a path to the file
          path = directory + id_num + filetype
          content = []
      ## Open the file
          with open(path,'r') as f:
          ## For every row in the file, split it by "," (comma
delimited), convert each value to a float
              for row in f:
                  row = row.split(","); row = [float(i) for i in row]
              ## if there are the correct number of entries (8, meaning
two objects have been detected), append it to a new list
                  if len(row) == 8:
                      content.append(row)
      ## convert to an array so we can do
          content=np.array(content)
          partner_data = []; r=[]; theta=[];
      ## Now we're going to go through all of the points and calculate
the distance and relative angle for each valid frame
          for row in content:
          ## Extract all of the relevant points
              pt1 = np.array(row[0],row[1]); a1 = row[2]; pt2 =
```

```
np.array(row[4],row[5]); a2 = row[6]
                dist = np.linalg.norm(pt1-pt2)
                rel_theta = 180 - abs(abs(a1-a2)-180)
                partner_data.append([dist,rel_theta])
            try:
                compiled = np.array(partner_data)
                compiled = calzone(compiled)
            except: print("problem with partner data")

        ## You now haven array, called "compiled", that contains the
distance and relative angle for all framesself.
        ## make some empty variables to count these
            follows = 0; orients = 0; disp = 0; coded = []
            for row in compiled:
                ## Check to see how close they are
                if row[0] <= dist_thresh:
                ## If they're close enough, compare the angles - if the
angle is similar enough, they're following, otherwise, they're orienting
                    if row[1] <= follow_thresh:
                        follows+=1; coded.append(1)
                    else: orients +=1; coded.append(2)
                ## and if they're too far away, they're dispersing
                else: disp +=1; coded.append(0)

            ## Now print frequencies of each
            ## try doing your own calculations on these!
            print(id_num +","+str(len(compiled))+","+str(follows)
+","+str(orients)+","+str(disp))
            ### This will provide the average over each column
        #    print(id_num + "," + str(np.nanmean(compiled,axis=0)))
        print(df)
```

Data Visualization:

```
#Written by Adeline Fecker
import seaborn as sns
import pandas as pd
import numpy as np
Data=pd.read_csv('~/Bi410/plot_data.csv') #import the raw data as a panda
Data.head() #check the file looks correct

###violin plot of all behavioral data
sns.set(style='white')
sns.set_context("paper")
plt= sns.catplot(x="Condition", y="Frequency",
             hue="Condition", col="Behavior",
             data=Data, kind="violin", palette='Set2',
             height=5, aspect=.7);
#figure = plt.get_figure()
#plt.savefig('ALLviolin.png', dpi=600)
#violin plot of light vs dark

sns.set(style='white')
sns.set_context("paper")
plt= sns.violinplot(x="Behavior", y="Frequency", hue="Vision",
                    data=Data, palette="Accent", split=True, size= (60,70),
                    scale="count", inner="quartiles")
```

```
plt.legend(loc='upper left')
#figure = plt.get_figure()
#figure.savefig('CTRLviolin.png', dpi=600)
###violin plot of control vs lateral line
sns.set(style='white')
sns.set_context("paper")
plt= sns.violinplot(x="Behavior", y="Frequency", hue="Lateral",
                    data=Data, palette="Pastel2", split=True,
                    scale="count", inner="quartiles")
plt.legend(loc='upper left')
#figure = plt.get_figure()
#figure.savefig('LLviolin.png', dpi=600)


####### Figures 4, 5 #########
import seaborn as sns
import pandas as pd
import numpy as np
from scipy.stats import pearsonr
import matplotlib.pyplot as plt
import numpy as np
from pingouin import pairwise_tukey
Data=pd.read_csv('~/Bi410/brain_data2.csv')
Data.head()
include= Data.groupby(['include']).get_group('yes')


#Total Intensity
sns.set_style("white")
sns.set_context("paper")
colors = ['#6fc276','#ff7fa7','#b790d4','#5a86ad']
sns.set_palette(sns.color_palette(colors))
plt=sns.swarmplot(x="condition", y="total_intensity",data=include,
                   color="black", size=7, edgecolor="gray")
plt=sns.violinplot("condition", "total_intensity", data=include,
                  size=7, inner=None, scale='width')
#plt.legend(['social', 'lone'], fontsize=10)
plt.set_xlabel("")
plt.set_xticklabels(["social ctrl", "lone ctrl",'social blind','lone blind'],
fontsize=12,rotation=40, ha='right')
plt.set_ylabel("total intensity", fontsize=14)
plt.set_aspect(0.000005)
plt.autoscale()
sns.despine()
#figure = plt.get_figure()
#figure.savefig('active_total.png', dpi=500, bbox_inches = "tight")


#Mean Intensity
sns.set_style("white")
sns.set_context("paper")
colors = ['#6fc276','#ff7fa7','#b790d4','#5a86ad']
sns.set_palette(sns.color_palette(colors))
plt=sns.swarmplot(x="condition", y="mean",data=include,
                   color="black", size=7, edgecolor="gray")
plt=sns.violinplot("condition", "mean", data=include, size=7, inner=None,
scale='width')
#plt.legend(['social', 'lone'], fontsize=10)
plt.set_xlabel("")
plt.set_xticklabels(["social ctrl", "lone ctrl",'social blind','lone blind'],
fontsize=12,rotation=40, ha='right')
plt.set_ylabel("mean intensity", fontsize=14)
plt.set_aspect(0.8)
plt.autoscale()
```

```
sns.despine()
#figure = plt.get_figure()
#figure.savefig('active_mean.png', dpi=500, bbox_inches = "tight")
```

Statistical Analysis:

```
import seaborn as sns
import pandas as pd
import pingouin as pg
import numpy as np
from pingouin import pairwise_tukey
orient=pd.read_csv('~/Bi410/file.csv')

print(orient.head()) #make sure file looks correct
anova = pg.anova(dv='Frequency', between = ['Vision','Lateral'],
data=disperse, detailed= True)
posthocs = pg.pairwise_ttests(dv='Frequency', between=['Condition'],
data=disperse, effsize='cohen')
pg.print_table(anova)
print(posthocs)
```

Example Merged Image:

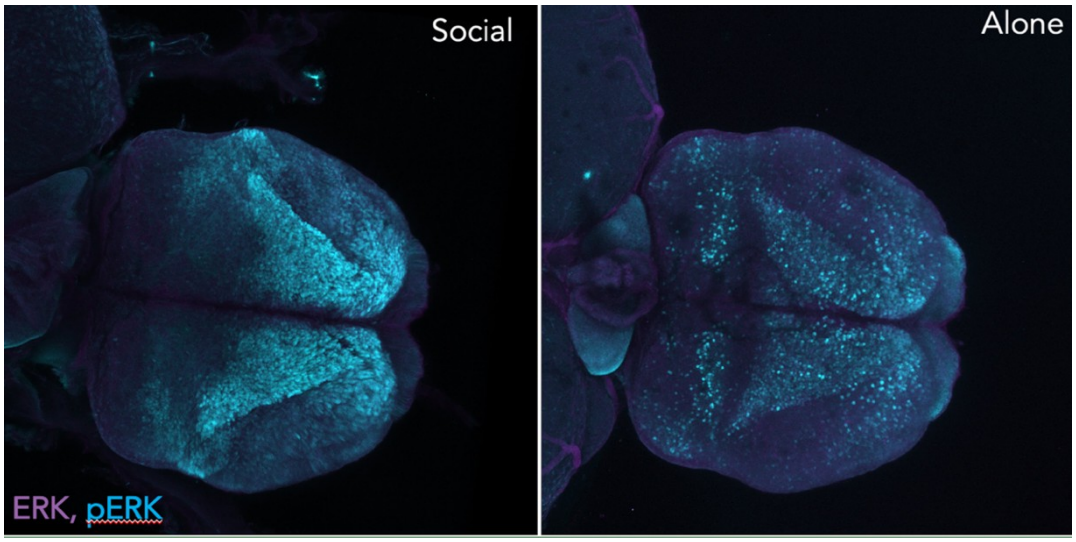


Figure from https://www.syngene.com/applications/fluorescence/

ERK protein and pERK protein can be recognized by primary antibodies from mice, rabbits, and other mammals. We can attach a secondary antibody by targeting it towards mice or rabbit primary antibodies. This secondary antibody contains a fluorophore that we can measure using a microscope. We can use different secondary antibodies to measure different proteins all on the same tissue.

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