Friedrich Schiller University Jena

Faculty of Biological Sciences

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Nonsynaptic cell-cell communication in the olfactory sensory system of *Drosophila melanogaster*

Master's Thesis

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List of Abbreviations

AL	Antennal lobe
D. melanogaster	*Drosophila melanogaster*
DA2	Dorso-anterior 2 (glomeruli)
DM2	Dorso-medial 2 (glomeruli)
DMV	Double Membrane Vesiscle
EM	Electron Microscope
FIB-SEM	Focused Ion Beam-Scanning Electron Microscope
FLAG	Polypeptide protein tag
FRT	Flippase Recognition Target
GAL4	yeast specific transcription factor
GCaMP	genetically encoded calcium indicator (GECI)
GFP	Green Fluorescent Protein
GH146	PN specific GAL4 driver line
HA	Human influenza hemagglutinin
hs-FLP	Heat-shock Flippase
LN	Local Interneuron
LSCM	Laser Scanning Confocal Microscope
LSFM	Light Sheet Fluorescence Microscope
MCFO	MultiColor FlpOut
myr	Myristoil
NGS	Normal Goat Serum
Or22a	Odorant receptor 22a
Or56a	Odorant receptor 56a

Or83b	Odorant receptor 83b
Orco	Odorant receptor coreceptor
ORN	Olfactory Receptor Neuron
OSN	Olfactory Sensory Neuron
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Triton X-100
PFA	Paraformaldehyde
PFAT	Paraformaldehyde with Triton X-100
PN	Projection Neuron
RFP	Red Fluorescent Protein
SEM	Scanning Electron Microscope
sfGFP	superfolder Green nonFluorescent Protein
smGFP	spaghetti monster Green Fluorescent Protein
UAS	Upstream Activation Sequence
V5	Peptide derived from the paramyxovirus of simian virus 5

Abstract

Besides synaptic communication, neurons can also interact via nonsynaptic communication. The neurobiology of *Drosophila melanogaster*, especially the chemosensory system, represents a very well described model. In this study, the DA2 and DM2 glomeruli, which are well documented as being highly involved in aversive and attractive behavior respectively in *D. melanogaster*, are investigated. Despite the discovery of a nonsynaptic form of communication via synaptic spinules with the aid of electron microscopy, a valid proof via light microscopy is still absent. All things considered, this study tries to visualize the synaptic spinules in the olfactory sensory system of *D. melanogaster* by employing a combination of genetic tools, such as GAL4/UAS system and MultiColor FlpOut with high-resolution fluorescence microscopy.

To differentially label pre- and postsynaptic neurons, two different approaches were applied. In the DA2 glomerulus, communication between olfactory sensory neurons was investigated by using the MultiColor FlpOut as a multicolor stochastic labeling technique. Many variables needed to be changed in pursuance of a standardization of the aforesaid technique. A heat shock timespan of eight minutes, as the most important variable, was shown to be the optimal time for an array of differently colored neurons in the DA2 glomerulus. The immunohistochemistry, the appropriate developmental stage of the fly, the genetics, the image acquisition were also crucial variables that were to be optimized. Protrusions in the olfactory sensory neurons were found, but a higher resolution is crucial to quantifying them. In the DM2 glomerulus, communication between olfactory sensory neurons and projection neurons was investigated by utilizing two different binary systems. The DM2 glomerulus was visible, but it was not possible to reveal synaptic spinules by using this technique, as the construction of a fly expressing both binary systems was burdensome. No signal was detected for the projection neurons, even though different antibodies were used to label them. In conclusion, a novel genetic protocol combined with high-resolution microscopy was established as a potential technique to further investigate synaptic spinules in the olfactory sensory system of *D. melanogaster*.

Zusammenfassung

Neben der synaptischen Kommunikation können Neuronen auch über nicht synaptische Kommunikation interagieren. Die Neurobiologie von *Drosophila melanogaster*, insbesondere das chemosensorische System, stellt ein sehr gut beschriebenes Modell dar. In dieser Studie werden die Glomeruli DA2 und DM2 untersucht, die in *D. melanogaster* als hoch involviert für aversives und attraktives Verhalten beschrieben sind. Trotz der Entdeckung einer nicht-synaptischen Form der Kommunikation über synaptische Spinula mit Hilfe der Elektronenmikroskopie fehlt noch ein stichhaltiger Nachweis mittels Lichtmikroskopie. Im Mittelpunkt dieser Studie steht die Visualisierung die synaptischen Spinula im olfaktorischen System von *D. melanogaster*, indem eine Kombination von genetischen Werkzeugen wie GAL4/UAS-System und MultiColor FlpOut mit hoch auflösende fluoreszierender Mikroskopie eingesetzt wird.

Um prä- und postsynaptische Neuronen differentiell zu markieren, wurden zwei verschiedene Ansätze angewendet. Im DA2-Glomerulus wurde die Kommunikation zwischen den olfaktorischen sensorischen Neuroronen untersucht, indem der MultiColor FlpOut als mehrfarbige stochastische Markierungstechnik verwendet wurde. Viele Variablen mussten im Zuge einer Standardisierung der vorgenannten Technik angepasst werden. Es wurde gezeigt, dass die achtminütige Zeitspanne des Hitzeschocks als die wichtigste Variable der optimale Zeitpunkt für eine Anordnung unterschiedlich gefärbter Neuronen im DA2-Glomerulus ist. Die Immunhistochemie, das geeignete Entwicklungsstadium der Fliege, die Genetik, die Bildaufnahme waren ebenfalls entscheidende Variablen, die optimiert werden mussten. Vorsprünge in den olfaktorischen sensorischen Neuronen wurden gefunden, aber eine höhere Auflösung ist entscheidend, um die Quantifikation zu ermöglichen. Im DM2-Glomerulus wurde die Kommunikation zwischen Riechsinneszellen und Projektionsneuronen untersucht, indem zwei verschiedene binäre Systeme verwendet wurden. Der DM2-Glomerulus war sichtbar, aber es war nicht möglich, synaptische Spinula mit dieser Technik zu enthüllen, da die Konstruktion einer Fliege, die beide binären Systeme exprimierte, mühsam war. Für die Projektionsneuronen wurde kein Signal detektiert, obwohl verschiedene Antikörper verwendet wurden, um sie zu markieren.

Zusammenfassend wurde ein neues genetisches Protokoll in Kombination mit hochauflösender Mikroskopie als potentielle Technik zu weiteren Untersuchungen von synaptischen Spinula im olfaktorischen System von *D. melanogaster* etabliert.

1. Introduction

1.1. Olfaction

Animals are susceptible to a large amount of chemical compounds. Avoidance of predators, evidence of a mating partner, detection of toxic chemicals, detection of food sources, and oviposition depend on the decoding of specific chemical compounds (Joseph and Carlson 2015). Olfaction is a chemosensory mechanism that aids the animal to cope with the changing external environment. Given a specific situation, olfaction provides the nervous system of the animal with enough information in favor of implementing the right behavioral response (Hansson and Stensmyr 2011). Processing and storage of perception regarding certain odors in the olfactory sensory system can be interpreted by knowing the types of cells that make up the system, their neural circuitry, and the way how these cells communicate with each other (Davis 2004).

1.2. *Drosophila melanogaster* as a model system

Drosophila melanogaster is an important model organism, because it has a short generation time and it is easy to be genetically manipulated. Their genome is completely sequenced (Adams, Celniker et al. 2000). In addition, fruit flies contain a considerable amount of genes with a human functional homolog. They generate many offspring; hence the outcome of a genetic cross can be investigated in a few weeks. Moreover, they have a low cost of maintenance (Wolf and Rockman 2008, Yamamoto, Jaiswal et al. 2014, Hales, Korey et al. 2015).

1.3. Binary expression systems

1.3.1. GAL/UAS system

The availability of the GAL4/UAS system is one of the advantages that *D. melanogaster* represents as a tool for different neurobiology studies. The GAL4/UAS is a binary expression system that allows the activation of a specific gene in different subsets of cells (Brand and Perrimon 1993). GAL4 is a transcription factor that regulates the galactose metabolism in *Saccharomyces cerevisiae*. When GAL4 binds to the upstream activation

sequence (UAS), the transcriptional activation of galactose genes takes place (Caygill and Brand 2016).

This system is based on two different lines: the activator line and the effector line. In this system, the expression of the protein occurs only in those tissues/cells that contain the specific driver. GAL4 acts as the transcriptional driver. Unless GAL4 binds to the UAS, the effector line will be transcriptionally silent (Brand and Perrimon 1993, Scheer and Campos-Ortega 1999, Grabe 2010). The GAL4 and UAS can both be expressed in the fruit fly with the aid of a simple genetic cross (**Figure 1**). The protein downstream the UAS gets transcribed only in those particular cells expressing the GAL4 transcriptional driver (Elliott and Brand 2008).

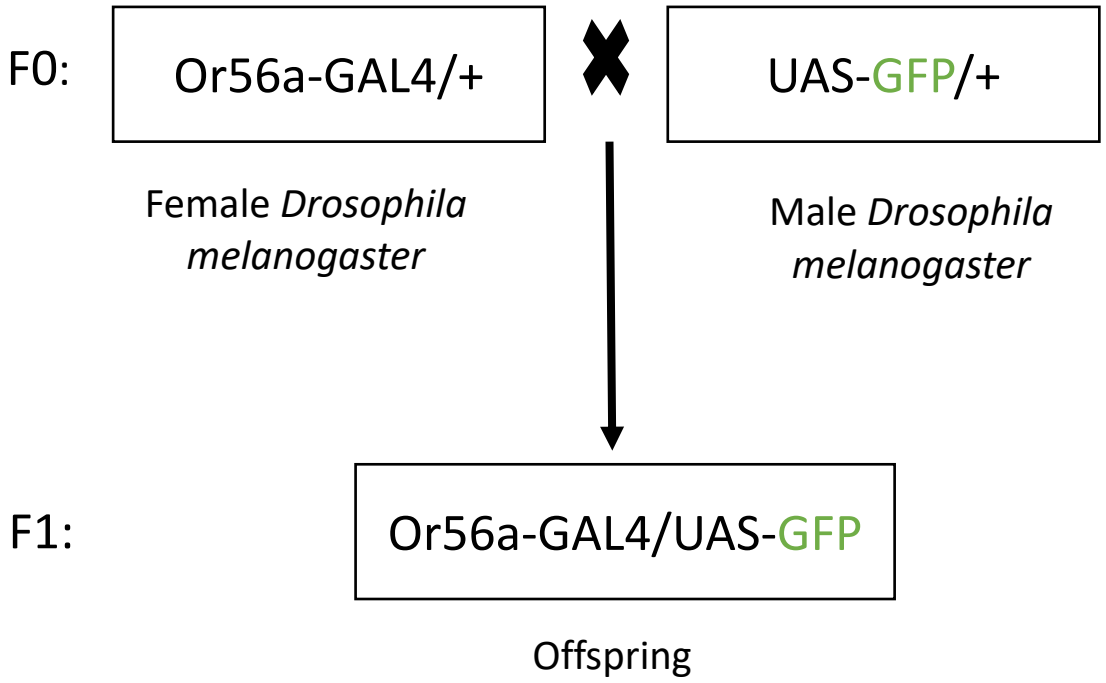

Figure 1. The GAL4/UAS binary expression system in the fruit fly *Drosophila melanogaster*. The specific transcriptional driver Or56a triggers the expression of the GAL4 protein in a subset of olfactory sensory neurons (OSNs). GAL4 protein binds to the UAS, which triggers the expression of the Green Fluorescent Protein (GFP) in the same subset of OSNs.

1.3.2. QF/QUAS system

In some experiments, except utilizing the GAL4/UAS system, one might need to apply other binary expression systems in order to differentially label other subsets of neurons. QF/QUAS binary system can also be used in *D. melanogaster*. QF acts in the same manner as GAL4, therefore it acts as a transcriptional driver. QUAS is analogous to UAS, thus functioning as the enhancer of the transcriptional driver (Brand and Perrimon 1993, Potter, Tasic et al. 2010).

1.4. Olfaction in *Drosophila melanogaster*

Odors are detected by two distinguishable organs, the maxillary palps and the antennae, especially the third antennal segment referred to as funiculus (**Figure 2**). They contain the olfactory receptor neurons (ORNs) (Stocker 1994). These neurons project axons to the first olfactory center, the antennal lobe (AL) of the brain, where they form synapses with different types of interneurons (Couto, Alenius et al. 2005, Joseph and Carlson 2015). Olfactory sensory neurons (OSNs) expressing the same olfactory receptor converge into one of the 52 differently shaped neuropils called glomeruli within the AL (Gao, Yuan et al. 2000, Grabe, Baschwitz et al. 2016). The majority of OSNs innervate ipsilateral and contralateral ALs (Stocker, Lienhard et al. 1990). After converging to the AL, the OSNs form synapses with local interneurons (LNs) and projection neurons (PNs) within different glomeruli (Rybak, Talarico et al. 2016).

OSNs of *D. melanogaster* express odorant receptor coreceptor 83b (Or83b), which is involved in dendritic localization of ligand-specific olfactory receptors, thus having a pivotal role in olfaction (Larsson, Domingos et al. 2004). These neurons also express other specific receptors, which are responsible for decoding of distinctive odors (Larsson, Domingos et al. 2004, Masse, Turner et al. 2009). Owing to the fact that Or83b (Orco) is restricly expressed in OSNs, labeling of the ALs can be carried out after immunohistochemistry of a fly brain that expresses the UAS-GFP enhancer, driven by the Orco-GAL4 transcriptional driver (**Figure 3**).

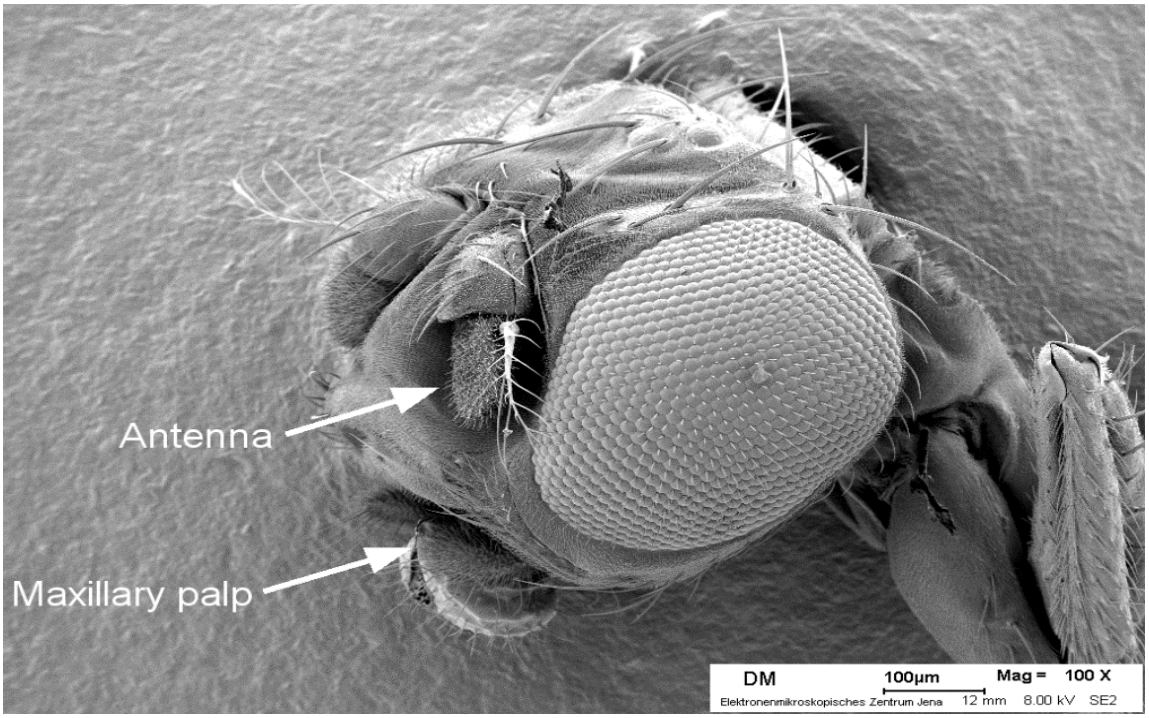

Figure 2. Lateral projection of the fore-head of *D. melanogaster* acquired with the aid of a Scanning Electron Microscope (SEM). Scale bar indicates 100 µm. Magnification (100X).

Figure 3. Frontal Laser Scanning Confocal Microscope (LSCM) projection of an adult fly brain. Immunohistochemistry with an anti-Bruchpilot antibody specific for neuropil (red) and anti-GFP antibody (green) on whole mount brains expressing UAS-GFP driven by Orco-GAL4. GFP is expressed in the ALs. Scale bar indicates 100 µm. Magnification (20X).

1.5. DA2 glomerulus

Geosmin (trans-1,10-dimethyl-trans-9-decalol) is a volatile compound that activates the OSNs expressing the odorant receptor 56a (Or56a) (**Figure 4**) (Stensmyr, Dweck et al. 2012). These neurons converge into the DA2 glomerulus and communicate with PNs by forming synapses (Stensmyr, Dweck et al. 2012). PNs project their axons from the DA2 glomerulus to higher olfactory centers, such as the mushroom body (MB) and the lateral horn (LH; also lateral protocerebrum), which process the olfaction response in favor of triggering an aversive behavior of the fruit fly (Jefferis, Potter et al. 2007, Stensmyr, Dweck et al. 2012, Grabe, Baschwitz et al. 2016).

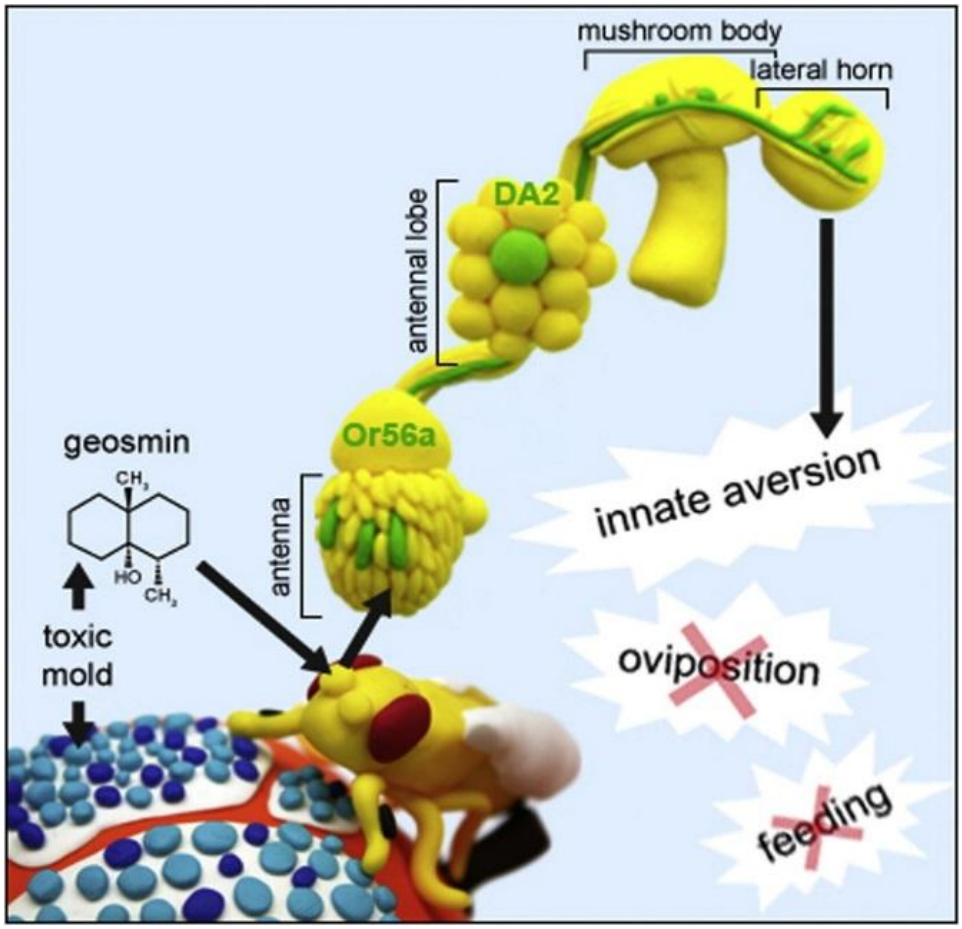

Figure 4. Circuit of olfactory neurons that gets triggered from geosmin to implement an avoidance behavior of the fruit fly (Stensmyr, Dweck et al. 2012).

By utilizing the Light Sheet Fluorescence Microscope (LSFM), one can carry out the imaging of the ALs after immunohistochemistry with an anti-GFP antibody of a whole mount brain of a transgenic fly expressing UAS-GFP driven by Orco-GAL4 (**Figure 5**).

Afterwards, the glomeruli can easily be recognized by its volume, shape and location (Grabe, Baschwitz et al. 2016).

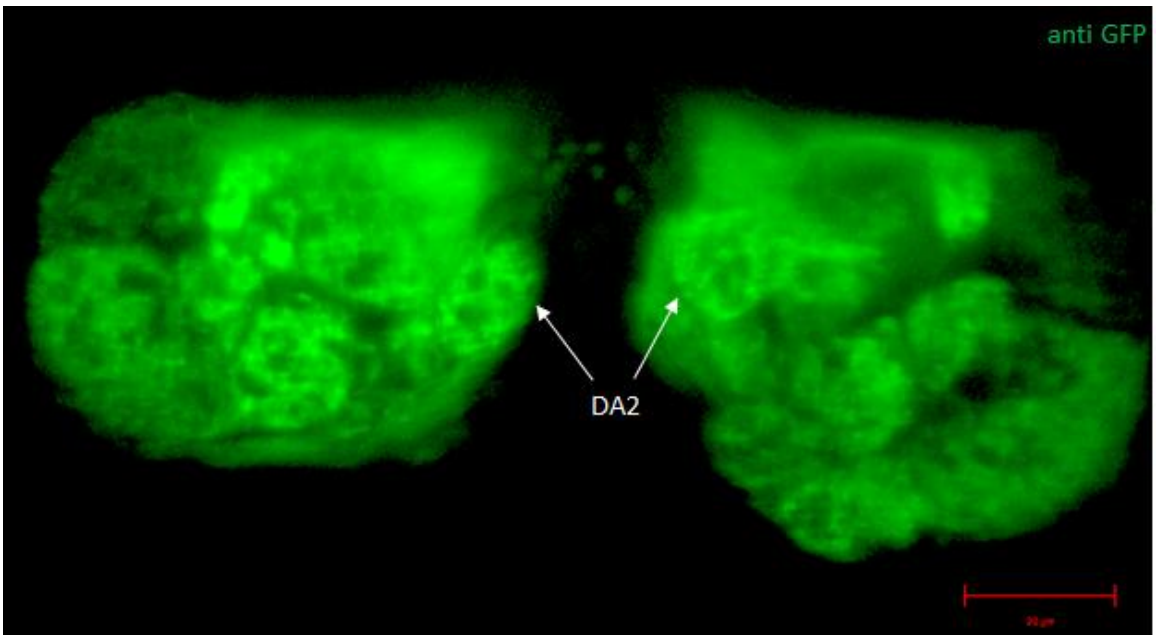

Figure 5. Frontal Light Sheet Fluorescence Microscope (LSFM) projection of the ALs of *D. melanogaster*. Immunohistochemistry with an anti-GFP antibody (green) of a whole mount brain of an adult fruit fly expressing UAS-GFP driven by Orco-GAL4. Scale bar indicates 20 µm. Magnification (20X).

1.6. DM2 glomerulus

Ethyl butyrate is an ester found in different fruits. Ethyl butyrate activates the OSNs expressing the odorant receptor 22a (Or22a). These neurons converge into the DM2 glomerulus and establish synapses with other OSNs, PNs, and LNs within the glomerulus (**Figure 6**) (Pelz, Roeske et al. 2006, Rybak, Talarico et al. 2016). PNs expressing the GH146 enhancer trap line innervate within the DM2 glomeruli (Shang, Claridge-Chang et al. 2007, Grabe, Baschwitz et al. 2016).

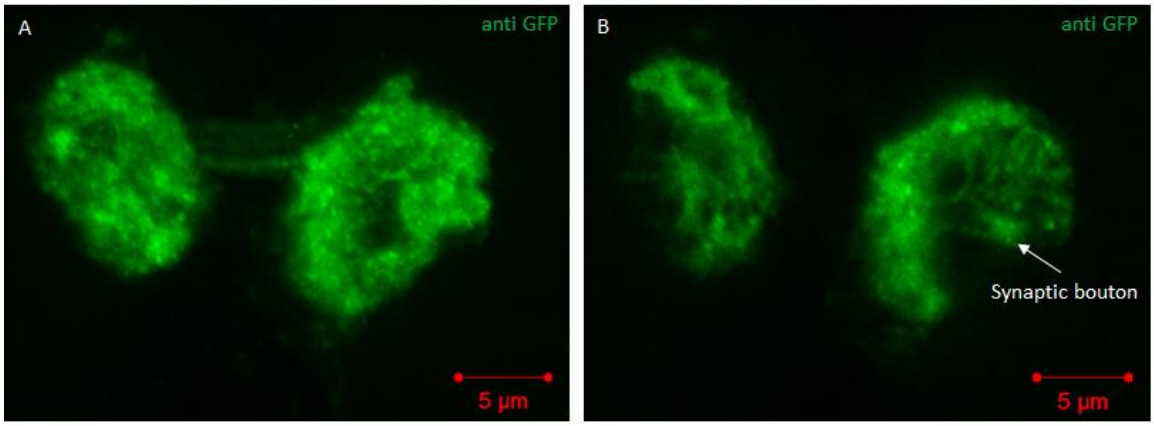

Figure 6. Frontal LSCM projection of the DM2 glomeruli of *D. melanogaster*. Immunohistochemistry with an anti-GFP antibody (green) of a whole mount brain of an adult fruit fly expressing UAS-GFP driven by Or22a-GAL4. **(A)** Maximum intensity projection of the DM2 glomeruli. **(B)** Frontal confocal projection of the DM2 glomeruli in a different depth. Synaptic bouton is visible. Magnification (63X).

1.7. Cell-cell communication

The most recognized way how neurons communicate with each other is by establishing chemical synapses. The neurotransmitter gets released from the presynaptic site and travels a very short distance through the synaptic cleft to reach the receptors that are located in the postsynaptic terminal of the adjacent neuron (Vizi and Kiss 1998, Vizi and Lendvai 2008).

A faster way of communication between neurons is the formation of electrical synapses, where the presynaptic neuron passes the electrical current through a gap junction in order to generate an action potential to the postsynaptic neuron (Hormuzdi, Filippov et al. 2004).

In addition to synaptic communication, neurons can also communicate through a distinct form of paracrine signaling, via invaginating projections (Petralia, Wang et al. 2015). The presynaptic membrane of one neuron invaginates and the postsynaptic membrane forms a protrusion into an invagination, hence establishing a form of paracrine signaling (Tarrant and Routtenberg 1977). These projections, shown to be invaginating the presynaptic terminals in the nervous system of mammals, are known as synaptic spinules (Tarrant and Routtenberg 1977, Petralia, Wang et al. 2015).

Following the discovery of synaptic spinules in mammals, evidence of nonsynaptic neuronal communication via synaptic spinules was also observed in the olfactory sensory system of *D. melanogaster* (Gruber, Rybak et al. 2018).

1.8. Synaptic spinules in *Drosophila melanogaster*

With the aid of electron microscopy (EM), protrusions in the presynaptic terminals of olfactory neurons and pinched-off materials referred to as double membrane vesicles (DMVs) were found across the ALs of *D. melanogaster*, specifically in the DA2 glomerulus (Spacek and Harris 2004, Gruber, Rybak et al. 2018). After reconstruction of olfactory neurons in the DA2 glomerulus by using an ImageJ plug-in, TrakEM2, mutually invaginating protrusions between OSNs were found (**Figure 7**) (Schindelin, Arganda-Carreras et al. 2012, Gruber, Rybak et al. 2018).

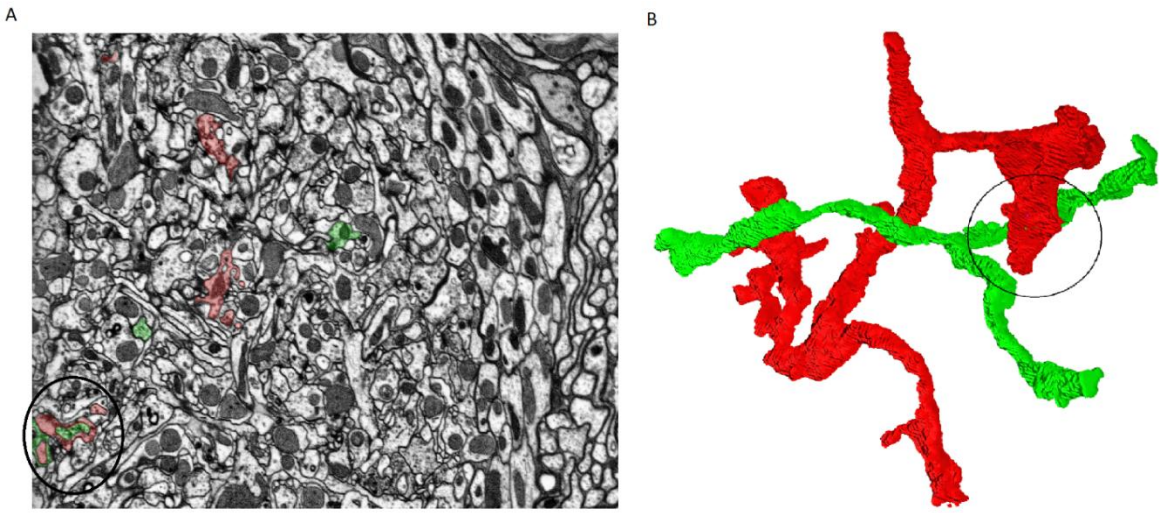

Figure 7. Single image acquired from Focused Ion Beam combined with Scanning Electron Microscopy (FIB-SEM) series across olfactory glomerulus DA2 of the fruit fly. (A) Two mutually invaginating protrusions between two OSN (red; green). **(B)** 3D-reconstruction of the two OSNs by using ImageJ (Gruber, Rybak et al. 2018).

1.9. MultiColor FlpOut (MCFO)

Recent studies have shown that the combination of light microscopy with single-color labeling eases the imaging of distinctive subsets of neurons and the reconstruction of the fly brain (Chiang, Lin et al. 2011, Nern, Pfeiffer et al. 2015). Further advances have been made for the brain of *D. melanogaster* by using different multicolor stochastic labeling techniques, such as Brainbow and Flybow (Hadjieconomou, Rotkopf et al. 2011, Hampel, Chung et al. 2011). Given the difficulty of controlling the labeling density in techniques such as Brainbow and Flybow, a new method called MultiColor FlpOut (MCFO) has been recently developed (**Figure 8**) (Nern, Pfeiffer et al. 2015).

The MCFO technique labels neurons in unique colors by using differently tagged reporters such as HA, FLAG, and V5, under UAS control. The immunoepitope tags are kept silent by two FRT transcriptional terminators (FRT-stop-FRT) (Nern, Pfeiffer et al. 2015, Au - Batelli, Au - Kremer et al. 2017). Subjecting the flies to a heat shock pulse expresses a heat shock-induced flippase (hs-FLP), which stochastically removes the stop cassettes in individual cells (Au - Batelli, Au - Kremer et al. 2017).

The immunoepitope tags are located into the backbone of a superfolder Green nonFluorescent Protein (sfGFP). The sfGFP is targeted to the plasma membrane of the cells by a N-terminal myristoylation signal (Pédelacq, Cabantous et al. 2005, Pfeiffer, Ngo et al. 2010, Nern, Pfeiffer et al. 2015). Put together, the expression of the immunoepitope tags is controlled by the transcriptional driver GAL4 and the stochastic removal of the FRT-stop-FRT cassettes (Nern, Pfeiffer et al. 2015).

In this study, the MCFO technique is used to produce an array of differently colored OSNs within the DA2 glomerulus, driven by the transcriptional driver Or56a-GAL4. Following immunohistochemistry, the immunoepitope tags could be detected due to the use of different antibodies, which emit signal in different wavelengths such as 488 nm, 546 nm, and 633 nm. As the expression of the immunoepitope tags is stochastic, OSNs inside the DA2 glomerulus can express up to seven different colors after immunoprocessing of the immunoepitope tags (**See Methods/Figure 9**) (Nern, Pfeiffer et al. 2015).

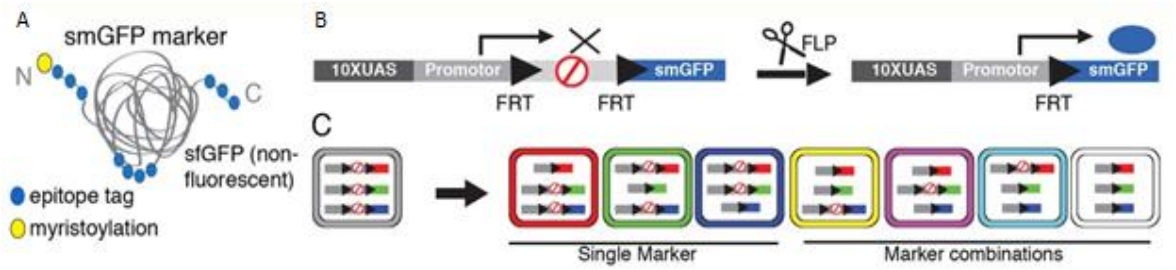

Figure 8. MultiColor FlpOut (MCFO) technique. (A) Spaghetti monster green fluorescent protein (smGFP). **(B)** MCFO reporter with the UAS-immunoepitope tags, transcriptional driver GAL4, FRT-stop-FRT cassette, and the hs-FLP (Nern, Pfeiffer et al. 2015).

1.10. Goals of the project

The aim of this thesis was to investigate nonsynaptic cell-cell communication in the olfactory sensory system of *D. melanogaster* by combining high-fluorescence microscopy with genetic tools such as GAL4/UAS system and the MCFO technique. Protrusions penetrating the presynaptic terminals of OSNs have been revealed in the ALs, specifically in the DA2 glomerulus by means of FIB-SEM (Gruber, Rybak et al. 2018). By differentially labeling the pre- and postsynaptic neurons, visualization of these protrusions by light microscopy was investigated. Therefore, the construction of transgenic flies expressing differentially labeled OSNs was a prerequisite for the experiments. Two major experiments were carried out. In the first experiment, hereinafter referred to as OSN-OSN experiment, communication between OSNs of the DA2 glomerulus was investigated. A novel technique called MCFO was predicted to stochastically label OSNs of the same glomerulus in different colors (Nern, Pfeiffer et al. 2015). In the second experiment, hereinafter referred to as OSN-PN experiment, communication between OSNs and PN in the DM2 glomerulus was investigated. A combination of two different binary systems (GAL4/UAS, QF/QUAS) was predicted to differentially label OSNs from PNs. Another goal of this thesis was to standardize the experimental conditions for using MCFO as a multicolor stochastic labeling technique for the DA2 glomerulus in the olfactory sensory system of *D. melanogaster*. The genetics, immunostaining of the olfactory neurons, the developmental phase of the fly, and the optimal timespan of the heat shock treatment were to be optimized.

2. Materials and Methods

2.1. Materials

2.1.1. Transgenic flies

Transgenic fruit flies such as: Orco-GAL4/UAS-GCaMp6s (Vosshall, Wong et al. 2000), Or22a-GAL4/UAS-CD8-GFP (Vosshall, Wong et al. 2000), Cyo/BL;GH146-QF,QUAS-mtdTomato/(TM6B) (Potter, Tasic et al. 2010) were used. For the MCFO experiments the following lines were used: Or56a-GAL4/Cyo (Vosshall, Wong et al. 2000) and 10XUAS-FRT-stop-FRT-myr-smGFP-HA,10XUAS FRT-stop-FRT-myr-smGFP-FLAG, 10XUAS-FRT-stop-FRT-myr-smGFP-V5 (Nern, Pfeiffer et al. 2015). Transgenic flies needed to be crossed in order to construct transgenic offspring flies expressing the effector gene controlled by the appropriate GAL4 driver (**Table 1**).

Table 1. List of the used transgenic flies

Fly line	Expressing neurons	Source
Or56a-GAL4	GAL4 under the Or56a promoter	BL 9988
MultColor FlpOut (MCFO)	Expresses HA and/or V5 and/or FLAG, each in a backbone of non-fluorescent myristoylated GFP under the control of UAS after removal of a stop cassette	FU Berlin MCFO-1 BL64085
Or22a-GAL4/UAS-GFP	Or22a expressing neurons	Vosshall/Silke
Orco-GAL4/UAS-GCaMp6s	Orco expressing OSNs	Kadow/Trautheim
QF-GH146/QUAS-mtdTomato	A subset of PNs	Bl 30037/Trautheim

2.1.2. Chemicals

Table 2. List of the used chemicals

Chemical	Dealer	Article Number
Calcium chloride	Roth, Karlsruhe, Germany	T881.3
Ethanol	Roth, Karlsruhe, Germany	9065.1
Glycerol	Roth, Karlsruhe, Germany	M92.1
HEPES	Sigma Aldrich, Steinheim, Germany	7365-45-9
Magnesium chloride	Sigma Aldrich, Steinheim, Germany	7791-18-6
Normal Goat Serum (NGS)	Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK	005-000-001
Paraformaldehyde	Roth, Karlsruhe, Germany	335.2
Potassium chloride	Roth, Karlsruhe, Germany	HN02.1
Sodium bicarbonate	Sigma Aldrich, Steinheim, Germany	S6014
Sodium chloride	Roth, Karlsruhe, Germany	9265.2
Sodium dihydrogen phosphate	Roth, Karlsruhe, Germany	T879.1
Sodium hydrogen phosphate	Roth, Karlsruhe, Germany	T877.2
Sodium hydroxide	Roth, Karlsruhe, Germany	9356.1
Sucrose	Sigma Aldrich,	57-50-1

	Steinheim, Germany	
Triton X-100	Sigma Aldrich, Steinheim, Germany	019K0151
Vectashield Antifade Mounting Medium	Vector Laboratories, Inc, Burlingame, USA	H-1000

2.1.3. Solutions

Table 3. List of the used solutions

Solution	Preparation method
10X PBS	76.0 g NaCl (1.3M) 0.94 g Na_2HPO_4 (0.07M) 3.6 g NaH_2PO_4 (0.03M) 1 l aqua bidest Adjust pH to 7.1 Autoclave for 1 hour at 121 ^{0}C
1X PBS	100 ml 10X PBS (stock solution 10X PBS) 900 ml H_20
1X PBS + 0.1% Triton X-100 (PBST)	5 ml 10X PBS 45 ml dH_20 50 µl Triton X-100
Blocking solution (PBST + 2% NGS)	50 ml PBST + 1 ml NGS
Drosophila's Ringer's solution	130 nM NaCl 2 nM $CaCl_2$ 5 nM KCl 2 nM $MgCl_2$ 36 nM Sucrose 5 nM Hepes-NaOH pH 7.4 Add 100 ml of dH_2O
Fixative solution (4% PFA)	Heat up 80 ml dH_20 in the microwave

	Add 4 g PFA and stir on the heating plate Add 10 µl 10N NaOH Add 10 ml 10X PBS Fill up to final volume of 100 ml Aliquot 1 ml and freeze at -20C
PFAT (4% PFA + 0.2% Triton)	2 µl Triton X-100 in 1 ml 4% PFA

2.1.4. Antibodies

2.1.4.1. Primary antibodies

Table 4. List of the used primary antibodies

Primary antibody	Dealer	Article number
Chicken anti-HA tag antibody	Sigma Aldrich, Steinheim, Germany	GW22511
Monoclonal mouse antibody nc82	Developmental Studies Hybridoma Bank, Iowa, USA	nc82
Mouse anti-GFP polyclonal antibody	Thermo Fisher Scientific, Darmstadt, Germany	A11120
Mouse anti-V5 tag antibody	Sigma Aldrich, Steinheim, Germany	V8012
Rabbit anti-FLAG tag antibody	Sigma Aldrich, Steinheim, Germany	F4725
Rabbit anti-GFP polyclonal antibody	Thermo Fisher Scientific, Darmstadt, Germany	A11122
Rabbit anti-RFP antibody	Abcam, Cambridge, UK	Ab62341

2.1.4.2. Secondary antibodies

Table 5. List of the used secondary antibodies

Secondary antibody	Dealer	Article number
Goat anti chicken Alexa Fluor 488	Thermo Fisher Scientific, Darmstadt, Germany	A11039
Goat anti chicken Alexa Fluor 633	Thermo Fisher Scientific, Darmstadt, Germany	A21103
Goat anti mouse Alexa Fluor 488	Thermo Fisher Scientific, Darmstadt, Germany	A11001
Goat anti mouse Alexa Fluor 546	Thermo Fisher Scientific, Darmstadt, Germany	A11030
Goat anti mouse Alexa Fluor 633	Thermo Fisher Scientific, Darmstadt, Germany	A21050
Goat anti rabbit Alexa Fluor 488	Thermo Fisher Scientific, Darmstadt, Germany	A11008
Goat anti rabbit Alexa Fluor 546	Thermo Fisher Scientific, Darmstadt, Germany	A11010

2.1.5. Equipments

Table 6. List of the used equipments

Equipment	Name	Dealer
Beaker	400 ml Borosilicate glass 3.3	VWR International, Radnor, PA
Centrifuge	MyFuge Mini	Biozym, Oldenforf, Germany
Cover glasses	Cover glasses thickness No.1	Paul Marienfeld, Lauda Koenigshofen, Germany
Coverlips	Menzel™ Microscope Coverslips	Thermo Fisher Scientific, Darmstadt, Germany
Digital dry bath	AccuBlock™	Labnet International Inc, Big Flats, NY

Electronic temperature controller	TC2	Imlab Bvba, Boutersem, Belgium
Fine forceps	Dumont #5	Fine Science Tools, Heidelberg, Germany
Kimwipes	Kimtech Science Precision Wipes	Kimerly-Clark Professional, Roswell, GA
Laser Scanning Confocal Microscope (LSCM)	ZEISS LSM 880 with Airyscan	Zeiss, Jena, Germany
Light Sheet Fluorescence Microscope (LSFM)	ZEISS Lightsheet Z.1	Zeiss, Jena, Germany
Magnetic stirrer with heating	MSC basic C	Imlab Bvba, Boutersem, Belgium
Microcentrifuge tubes	0.5 ml Eppendorf tubes	Eppendorf, Hamburg, Germany
Microscope slides	HistoBond® + M	Paul Marienfeld, Lauda-Koenigshofen, Germany
Minutien pins	No. 26002-10	Fine Science Tools, Heildeberg, Germany
Nutator	Polymax 1040	Heidolph, Schwbach, Germany
Paintbrush	Superfine Eyelash #113	Ted Pella Inc, Redding, CA
Pipettes	Pipetman	Gilson, Middleton, WI
Stereo Microscope	SZX16	Olympus, Tokyo, Japan
Vortexer	Vortex Genie 2	Scientific Industries, Bohemia, NY

2.1.6. Softwares

Table 7. List of the used softwares

Software	Dealer
Adope Photoshop CS	Adobe Systems
Amira 5.3	Fei, Visualization Science Group
EndNote X8	Clarivate Analytics
Image J	Public Domain
Microsoft Office	Microsoft Corporation
Microsoft Paint	Microsoft Corporation
ZEISS ZEN 2 Imaging Software	Zeiss, Jena, Germany

2.2. Methods

2.2.1. Animal rearing

Flies were raised in an incubator at 25 ^{0}C and 70% humidity with 12 hours' day and night cycle. Adult flies were flipped to new vials every two weeks. In the experiments where the communication between OSNs and PNs was investigated, female flies were dissected five days after eclosion. In the experiments where the communication between OSNs of the same glomerulus was investigated, female flies of different ages were used. Larvae, pupae, one day old flies, and five day old flies were used. This was carried out in order to establish the appropriate timepoint during the development stages of the fly, so the latter could be subjected to a successful heat shock treatment.

2.2.2. Crossing and construction of transgenic flies

In the MCFO experiment, nonsynaptic communication between OSNs of the DA2 glomerulus was investigated. Therefore, virgin female flies carrying the enhancer i.e. MCFO cassete (10XUAS-FRT-stop-FRT-myr-smGFP-HA,10XUAS-FRT-stop-FRT-myr-smGFP-FLAG, 10XUAS-FRT-stop-FRT-myr-smGFP-V5) and the hs-FLP were crossed with male flies carrying Or56a-GAL4 transcriptional driver (**Figure 9**). Following immunohistochemistry, the OSNs of the DA2, labeled in a maximum of seven different colors, could be visualized by light microscopy.

In the other experiment, nonsynaptic communication between OSNs and PNs of the DM2 glomerulus was investigated. Therefore, virgin female flies carrying the transcriptional driver Or22a-GAL4 and the enhancer UAS-CD8-GFP were crossed with male flies carrying the transcriptional driver GH146-QF and the enhancer QUAS-mtdTomato (**Figure 10**).

The offspring flies contained two different binary systems (Or22a-GAL4/UAS-CD8-GFP, GH146-QF/QUAS-mtdTomato). Following immunohistochemistry, two different subsets of neurons such as the OSNs of the DM2 and a subset of uniglomerular PNs could be visualized by light microscopy.

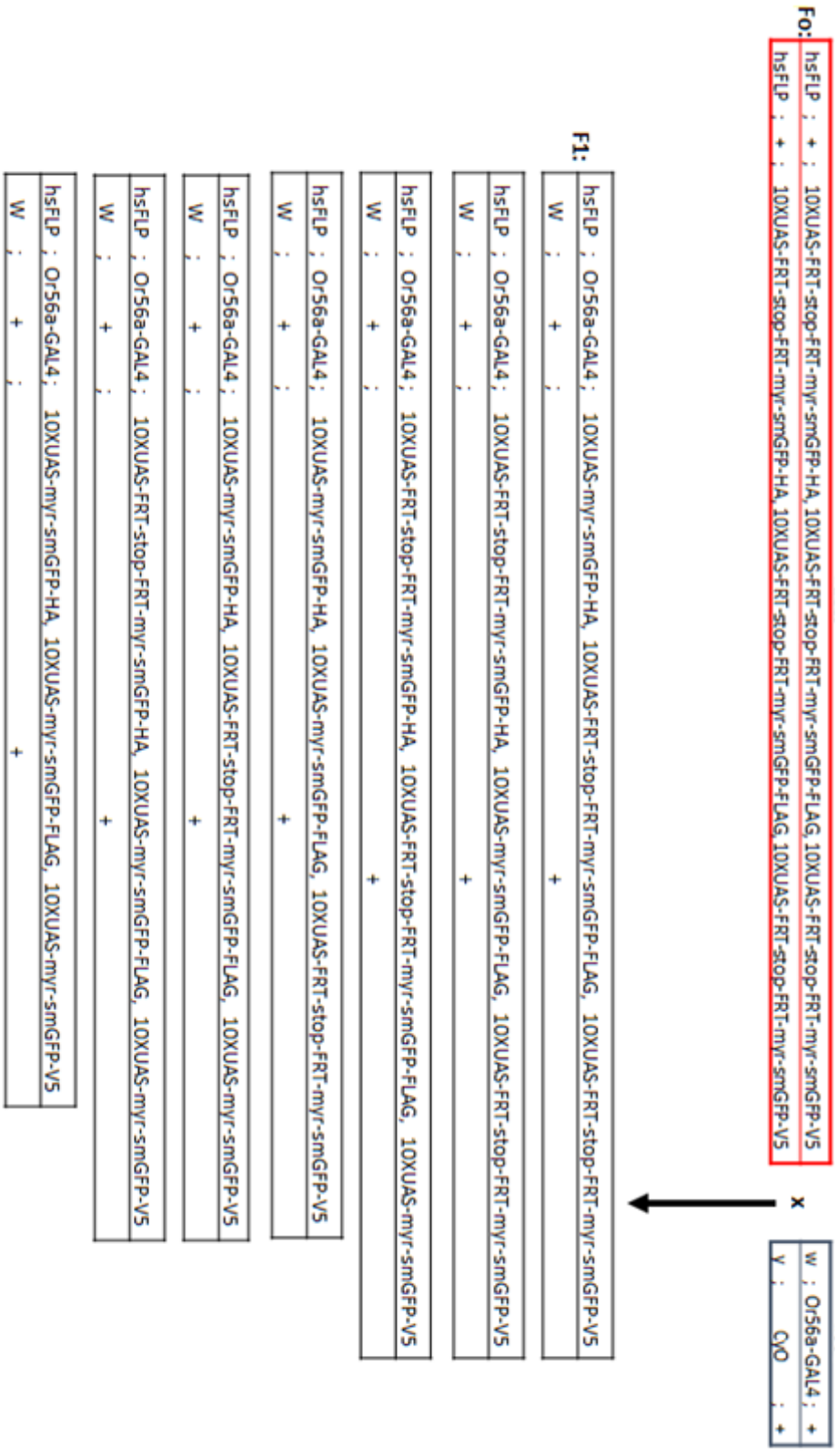

Figure 9. Crossing scheme of the MCFO virgin female flies with male flies expressing the transcriptional driver Or56a-GAL4.

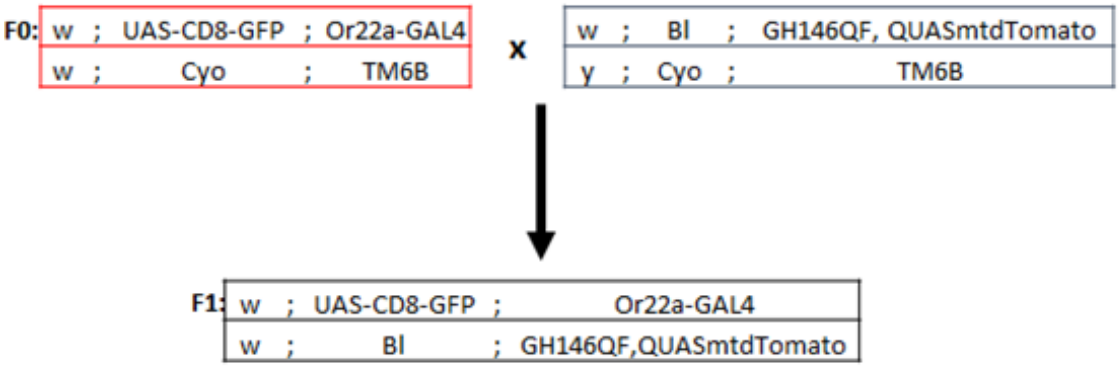

Figure 10. Crossing scheme of virgin female transgenic flies expressing UAS-CD8-GFP driven by the Or22a-GAL4 with male transgenic flies expressing QUAS-mtdTomato driven by the transcriptional driver GH146-QF.

2.2.3. Heat shock

After crossing the flies, the offspring flies of the F1 generation (**Figure 9**) were subjected to a heat shock treatment two days after eclosion. Following the heat shock treatment, the OSNs of the DA2 glomerulus of the offspring flies could express up to seven different colors after immunoprocessing of the immunoepitope tags, because the FRT-stop-FRT cassettes could randomly get excised by an active hs-FLP (**See Introduction/Figure 8**). Some flies could have only one cassete removed, thus expressing only one particular immunoepitope tag. Some flies could have two stop cassettes removed and some flies could have all three stop cassettes removed, hence expressing all three immunoepitope tags (Nern, Pfeiffer et al. 2015).

The offspring of the MCFO experiment were divided into new vials containing only female or male flies. Afterwards, flies were subjected to a heat shock of 37 ^{0}C in a water bath (**Figure 11**). Larvae and pupae were also subjected to heat shock treatments in separate vials in order to optimize the right developmental stage of the fly that would generate the best reporter expression. To subject the flies to a heat shock treatment, a magnetic stirrer with heating equipped with an electronic temperature controller (Imlab Bvba, Boutersem, Belgium) was used (**Figure 11**). The vial was submerged inside a 400 ml beaker (VWR International, Radnor, PA) containing 200 ml of water. The water inside the beaker was heated until 37 ^{0}C and the vial containing flies was immersed inside the beaker for different timespans, such as 20 min, 15 min, 8 min, and 5 min. The electronic temperature controller was used to set and keep the temperature at 37 ^{o}C. By submerging

the entire vial inside the beaker, one could be ensured that the heat shock treatment would be homogenous to all the flies inside the vial (Au - Batelli, Au - Kremer et al. 2017). Immediately after the heat shock treatment, the flies were kept horizonally on the bench for 15 minutes. Flies need some time to recuperate from the heat shock treatment, because 37 ^{0}C represents a high temperature for *D. melanogaster* (Au - Batelli, Au - Kremer et al. 2017). Subsequently, flies were placed in an incubator at 25 ^{0}C and 70% humidity with 12 hours day and night cycle. In order to optimize the MCFO technique, flies were dissected immediately, after one day, and after five days to find out the appropriate time needed for the hs-FLP to be translated and to properly excise the FRT-stop FRT cassettes. Following the heat shock treatment, flies were dissected, immunostained and scanned via LSM 880 with Airyscan (Carl Zeiss, Jena, Germany).

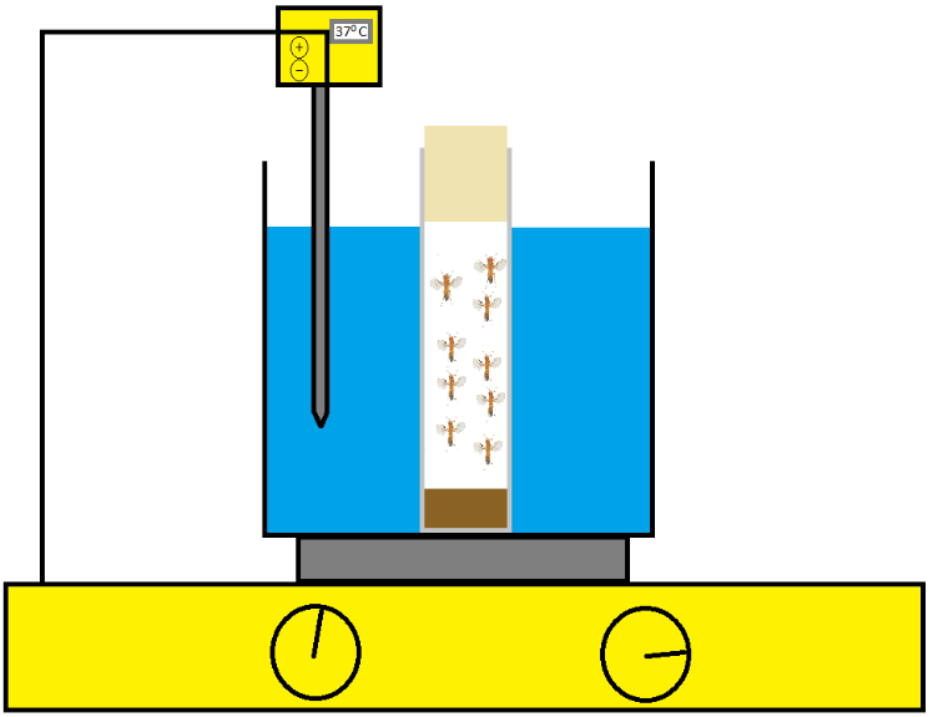

Figure 11. Heat shock setup. The vial was immersed inside the beaker and the water was heated until 37 ^{0}C by using a a magnetic stirrer with heating. The temperature was controlled by using an electronic temperature controller (https://www.cam.ac.uk/sites/www.cam.ac.uk/files/inner-images/150626-fruit-fly2.jpg).

2.2.4. Immunohistochemistry

First, the adult flies were separated in smaller vials and anaesthetized for at least 10 minutes by placing them on ice. Afterwards, flies were dissected as described in (Wu and Luo 2006) with minor differences. Flies were picked up from the vial by using a paintbrush (Ted Pella Inc, Redding, CA) and they were placed on the dissection dish (Petri dish lines at the bottom with 5 mm of Sylgard 184, World Precision Instruments, Cat#: SYLG184) on an individual basis. The SZX160 Stereo Microscope (Olympus, Tokyo, Japan) with parfocal objectives (0.5X, 1.0X) was used for the dissection of the brains of *D. melanogaster*. Ice-cold *Drosophila*'s Ringer solution was used to completely cover the fly. Afterwards, two fine forceps (Dumont #5, Fine Science Tools, Heidelberg, Germany) were used to dissect the brains and to remove all the tracheal tissue. Same procedure was applied to all the flies (Williamson and Hiesinger 2010).

Subsequently, the fly brains were collected in a 500 µl microcentrifuge tube (Eppendorf, Hamburg, Germany). Phosphate Buffered Saline (PBS) was added inside the tube, so the brains would not dry out. The tip of a P1000 pipette (Pipetman, Middleton, WI), was used to transfer the dissected brains from the dissection dish to the microcentrifuge tube. To ensure that the brains were never in contact with air during the transfer, the tip of the P1000 pipette needed to be rinsed and filled with PBS. Prior to immunohistochemistry, 10-15 flies were dissected for each experiment.

After collecting all the brains from the offspring of the F1 generation (**See Methods/Figure 9, 10**) that were needed for the experiment in one microcentrifuge tube, PBS was removed and replaced with 500 µl of ice-cold fixative solution (4% paraformaldehyde + 0.2% Triton X-100 (PFAT)). The microcentrifuge tube was incubated on a nutator (Polymax 1040, Heidolp, Schwbach, Germany) for 30 min. Next, the fixative solution was replaced with 500 µl of washing solution (Phosphate Buffed Saline + 0.1% Triton X-100 (PBST)). The brains were rinsed at least three times for 15 minutes at 4 ^{0}C with the washing solution. The latter was replaced with 500 µl of blocking solution (PBST + 2% Normal Goat Serum (NGS)) and incubated for 40 min rotating on a nutator at room temperature. The blocking solution was carefully removed, leaving only a small volume covering the brains.

To label the HA, FLAG, V5, GFP, mtdTomato, and nc82, different primary and secondary were used. After fixing and blocking, the brains were subjected to primary antibobody treatment. Primary antibodies (chicken anti-HA tag (Sigma Aldrich, GW22511), rabbit anti-FLAG tag (Sigma Aldrich, F4725), mouse anti-V5 tag (Sigma Aldrich, V8012)) were used in a 1:250 concentration. Additionally, rabbit anti-RFP (Abcam, ab62341) and mouse anti-GFP (Thermo Fisher Scientific, A11120) were used in a 1:500 concentration. Monoclonal mouse nc82 (Developmental Studies Hybridoma Bank, nc82) was used in a 1:30 concentration. Additionally, different concentrations (1:200/1:250/1:300/1:500) were tried to optimize the MCFO immunohistochemistry protocol. The primary antibodies were diluted in 500 µl of blocking solution. The brains were incubated in primary antibody solution for two nights rotating on a nutator at 4 ^{0}C. Afterwards, the brains were washed five times with 500 µl of washing solution for 15 min each at room temperature. Next, the washing solution was replaced with 500 µl of blocking solution for 30 min rotating at room temperature. Lastly, the blocking solution was discarded.

The secondary antibodies (1:250 goat anti-chicken Alexa Fluor 488 (Thermo Fisher Scientific, A11039), 1:250 goat anti-rabbit Alexa Fluor 546 (Thermo Fisher Scientific, A11010), 1:250 goat anti-mouse Alexa Fluor 633 (Thermo Fisher Scientific, A21050), 1:250 goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, A11001) and 1:250 goat anti-rabbit Alexa Fluor 546 (Thermo Fisher Scientific, A11010) were diluted in 500 ml of blocking solution. Subsequently, the brains were incubated in secondary antibody solution overnight rotating on a nutator at 4 ^{0}C. The microcentrifuge tubes were wrapped with aluminium foil before incubation to be protected from natural light. Lastly, the brains were washed five times with PBS for 15 min each at room temperature. PBS was used instead of PBST as a washing solution to remove the traces of Triton X-100 for a better resolution during image acquisition.

In addition, different secondary antibodies were tried in order to optimize the MCFO immunohistochemistry protocol. Goat anti-chicken Alexa Fluor 633 (Thermo Fisher Scientific, A21103), goat anti-mouse Alexa Fluor 546 (Thermo Fisher Scientific, A11030), and goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, A11008) were also used. Moreover, different concentrations (1:100/1:200/1:300/1:500) for different secondary antibodies and different incubation times were also carried out until standardization of the MCFO immunohistochemistry protocol.

To mount the brains, a spacer was built on a slide (Paul Marienfeld, Lauda Koenigshofen, Germany) by using coverslips (Thermo Fisher Scientific, Darmstadt, Germany). The coverslip spacer was sealed with nail polish and the brains were placed in between the spacer at the acquired position. The washing solution was removed with a P10 pipette (Pipetman, Middleton, WI) and replaced with a drop of Vectashield Antifade Mounting Medium (Vector Labs Inc., Burlingame, USA). A coverslip was placed over the brains. The excess of the Vectashield solution was removed and the border of the coverslip was sealed with nail polish. The sample needed to be covered for 30 min at room temperature and afterwards it was either scanned via fluorescence microscopy or stored at 4 ^{0}C.

2.2.5. Imaging

2.2.5.1. Light Sheet Fluorescence Microscopy

Lightsheet image stacks were acquired by using a ZEISS Lightsheet Z.1 (Carl Zeiss, Jena, Germany) with a 20 X water immersion objective (Plan-Apochromat, NA: 1.0 UV-VIS_4909220075, Carl Zeiss). For detection of the GFP in the ALs of *D. melanogaster*, the Argon 488-nm laser was used. A filter that detected the emission spectrum of 505-545 nm was used.

2.2.5.2. Laser Scanning Confocal Microscope

Confocal image stacks were obtained by using a ZEISS LSM 880 with Airyscan (Carl Zeiss, Jena, Germany) with a a 20 X water immersion objective (Plan-Apochromat, NA: 0.8, Carl Zeiss), a 40 X water immersion objective (C-Apochromat, NA: 1.20, Carl Zeiss), and a 63 X glycerol immersion objective (LCI Plan-Neofluar, NA: 1.3, Carl Zeiss).

The pinhole aperture of the LSM was set at one Airy unit for all the experiments. Argon 458, 477, 488, 514 nm, He/Ne 543, and He/Ne 633 lasers were used to visualize the immunostained samples. For the OSN-OSN experiment different tracks of filters were used, such as 490-530, 550-620, and 638-700 nm. Three different filters were used for in order to detect the immunoepitope tags. Separate confocal stacks were obtained for each immunoepitope tag. For the OSN-PN experiments a filter range of 470-586 nm was used to detect GFP, nc82, and mtdTomato.

2.2.5.3. Image processing

Amira 5.3 (Fei, Visualization Science Group), Image J (Public Domain), ZEISS ZEN 2 Imaging Software (Zeiss, Jena, Germany), and Adobe Photoshop CS (Adobe Systems) were used for adjustment of the confocal image stacks for contrast and brightness, changes in channel hue, maximum intensity projections, and rotations.

3. Results

3.1. OSN-OSN experiment

In order to visualize the interaction between OSNs expressing the same olfactory receptor and converging to the same glomerulus, the MCFO technique was used to stochastically label OSNs of the DA2 glomerulus.

3.1.1. Construction of the experimental flies

Flies expressing the hs-FLP, immunoepitope tags-UAS, and the Or56a-GAL4 transcriptional driver, were a prerequisite to the standardization of the MCFO protocol. Consequently, a combination of transgenic flies through classic genetic crosses had to be carried out (**See Methods/Figure 9**). This was achieved by implementing a classic genetic cross between virgin female flies expressing the hs-FLP in the first chromosome and the immunoepitope tags-UAS in the third chromosome with male adult flies expressing Or56a-GAL4 in the second chromosome. Consclusively, transgenic flies expressing hs-FLP in the first chromosome, Or56a-GAL4 in the second chromosome, and the immunoepitope tags-UAS in the third chromosome were successfully constructed.

3.1.2. Standardization of the MCFO technique

Different variables of the MCFO technique were adjusted for a sparse labeling of the OSNs of the DA2 glomerulus in the chemosensory system of the fruit fly. It is predicted that nonsynaptic communication via synaptic spinules could be visualized by light microscopy, only if the adjacent OSNs would be immunostained with different fluorephores (Gruber, Rybak et al. 2018). Even though MCFO labels a population of cells in a stochastic manner by randomly removing the stop cassettes, some variables such as the immunohistochemistry, the right developmental stage of the fly, the genetics, and the heat shock timespan would possibly differ the outcome the outcome of the MCFO technique in the chemosensory system of the fruit fly. For the aforesaid reasons, different timespans of heat shock pulses were executed in pursuance of the optimization of the appropriate heat shock timespan to differentially label adjacent OSNs in the DA2 glomerulus of *D. melanogaster*. Several timespans such as 20 minutes, 15 minutes, 8 minutes, and 5 minutes were investigated.

Furthermore, experiments with flies that were not subjected to a heat shock pulse were carried out to reveal if the heat shock was indeed responsible for the expression of the immunoepitope tags in the olfactory sensory system of the fruit fly. Afterwards, flies were dissected five days after eclosion and immediately after eclosion to check if the hs-FLP required a few days to be transcribed and translated from the fruit fly.

3.1.2.1. Heat shock treatment of 20 min to larvae and pupae

First, pupae and larvae from the same vial of the F1 generation (**see Methods, Figure 9**) were subjected to a heat shock pulse of 20 min. The timespan of 20 min was chosen based on similar experiments that were carried out to the optical lobe and the glial cells of *D. melanogaster* (Nern, Pfeiffer et al. 2015, Kremer, Jung et al. 2017). After immunostaining of the fly brains with three different antibodies (anti-HA, anti-FLAG, anti-V5) to detect the immunoepitope tags, no signal was detected at the DA2 glomerulus (**Figure 12 A**). The same results were obtained by 10 other flies dissected immediately after eclosion (**Data not shown**). Additionally, no signal was detected from flies of the same vial, dissected five days after eclosion (**Figure 12 B**).

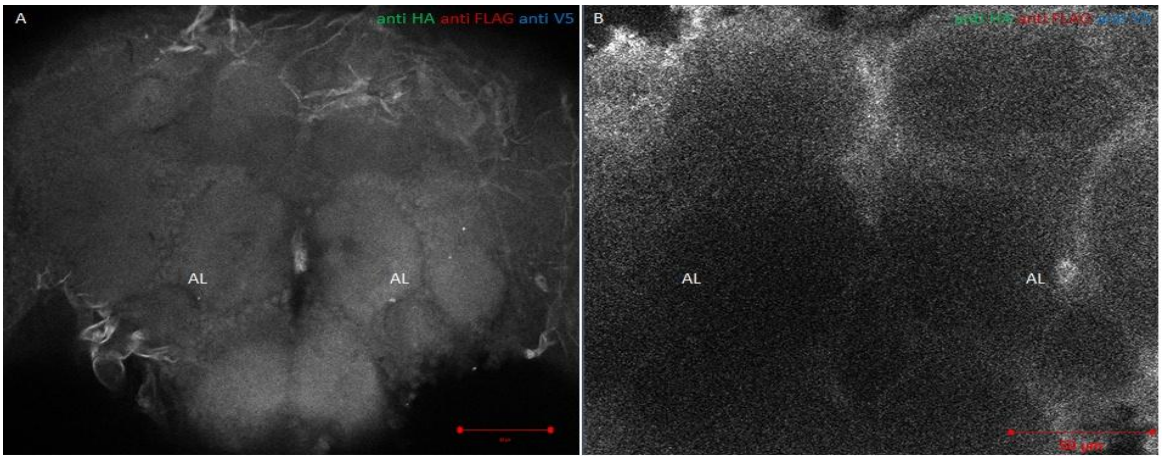

Figure 12. Frontal confocal projections of the female adult brain dissected immediately (A) and five days after eclosion (B). Flies were heat shocked for 20 min during larval stage. No signal could be detected at the ALs after immunohistochemistry (anti-HA, anti-FLAG, anti-V5). Both scale bars indicate 50 µm. Magnification (20X).

The heat shock pulse appears to have not induced the transcription of the hs-FLP during larval and pupal developmental stages. Or56a may be expressed later during the developmental stage of the fly. Therefore, the GAL4 transcriptional driver might have not been translated in the OSNs of the DA2 glomerulus, stalling the accumulation of the immunoepitope tags. After revealing that the heat shock treatment of 20 min could not induce the expression of the immunoepitope tags, experiment with adult flies were carried out.

3.1.2.2. Heat shock treatment of 20 minutes to adult flies

Female adult flies, dissected three days after being subjected to a 20 min heat shock pulse, revealed a co-expression of the three immunoepitope tags (HA, FLAG, V5) in the same OSNs of the DA2 glomerulus (**Figure 13 A, B, C**). Synaptic boutons or areas of presynaptic terminals could be visualized by means of confocal microscopy. However, the interaction of adjacent OSNs could not be visualized because of the co-expression of the same immunepitope tags.

After processing the acquired images, only OSNs expressing the Or56a appear to have been immunostained (**Figure 13**). The heat shock pulse of 20 min appears to have induced the transcription and the translation of the hs-FLP in order to flank all the three FRT-stop-FRT sites in all of the OSNs, hence expressing the immunoepitope tags in the same manner at the membrane of the OSNs of the DA2 glomerulus (**Figure 13 D**).

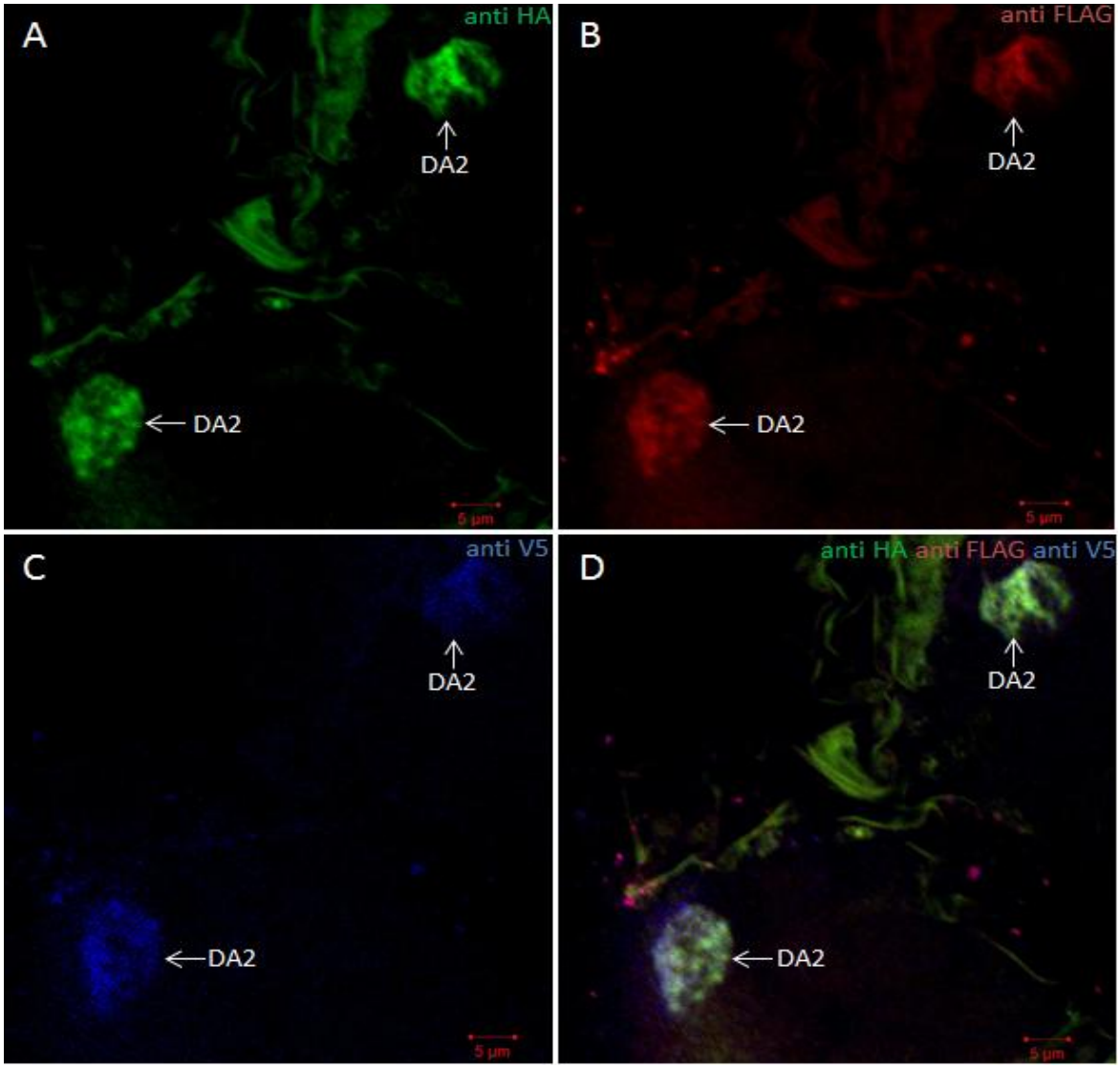

Figure 13. Frontal confocal projections of the DA2 glomeruli after 20 min of heat shock pulse. Co-expression of the three immunoepitope tags (HA. FLAG, V5) in the same OSNs of the DA2 after immunohistochemistry (anti-HA, anti-FLAG, anti-V5). **(A)** DA2 glomeruli labeled by anti-HA antibody (green). **(B)**. DA2 glomeruli labeled by anti-FLAG antibody (red). **(C)** DA2 glomeruli labeled by anti-V5 antibody (blue). **(D)** Merged image from **(A)**, **(B)**, and **(C)**. Scale bar indicates 5 µm. Magnification (20X) .

To further enhance the resolution, different concentrations of the primary antibodies were tried. A better resolution during image acquisition was achieved when a concentration of 1:250 was used instead of 1:200, 1:300, 1:500 (**Data not shown**).

Furthermore, different types of secondary antibodies were used. Goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa Fluor 546, and goat anti-mouse Alexa Fluor 633 appeared to have immunostained the samples better than the other secondary antibodies (**Data not shown**).

3.1.2.3. No heat shock treatment

Rearing the flies at the 25 ^{0}C incubator could also induce the expression of the hs-FLP at a low activity level, thus expressing the immunoepitope tags in a few OSNs after a few days. To assess if the hs-FLP gets activated explicitly after the heat shock treatment (37 ^{0}C) during adult stage, female flies from the F1 generation of the genetic cross (**see Methods, Figure 9**) were dissected immediately after eclosion. A translated and active hs-FLP would indicate that the excision of the FRT-stop-FRT sites occurs also without the heat shock pulse, thus pointing out that the latter is not pivotal for the labeling of the OSNs in the fruit fly.

Subsequent to immunohistochemistry of the brains of the female adult flies dissected immediately after eclosion with three antibodies (anti-HA, anti-FLAG, anti-V5), no signal was detected at the DA2 glomeruli. The AL showed no signal either (**Figure 15**). To check the level of activation of the hs-FLP at 25 ^{0}C female adult flies were dissected five days after eclosion. After carrying out the same immunohistochemistry principle to the aforementioned flies, the same results were obtained. Neither DA2, nor AL appeared to generate signal (**Data not shown**).

In conclusion, the heat shock treatment appears to be crucial for the removal of the FRT-stop-FRT cassettes and expression of the immunoepitope tags in such a way that the latter could accumulate and be detectable by means of light microscopy.

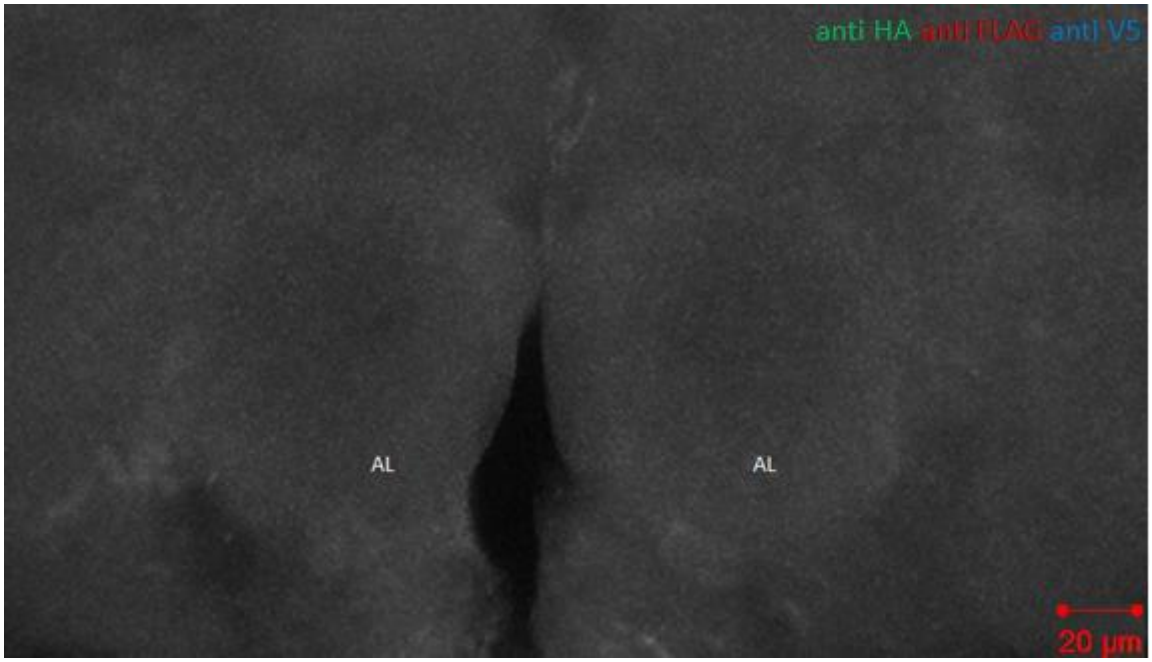

Figure 14. Single confocal image of the female adult brain dissected five days after eclosion without being subjected to a heat shock pulse. No signal was detected at the ALs after immunohistochemistry (anti-HA, anti-FLAG, anti-V5). Scale bar indicates 20 µm. Magnification (20X).

3.1.2.4. Heat shock treatment of 15 minutes

A heat shock pulse of 20 min showed a co-expression of the three epitope tags in the OSNs of the DA2 glomerulus (**Figure 14**), thus a shorter time frame of the heat shock pulse was tried in order to generate more differentially immunostained OSNs and less co-expression of the epitope markers in adjacent OSNs. For that reason, a heat shock of 15 min was carried out. Female adult flies from the F1 generation (**See Methods, Figure 9**), dissected three days after being subjected to a heat shock pulse of 15 min, showed a co-expression of two epitope tags (HA, FLAG) at the DA2 glomeruli. Interestingly, V5 was not expressed (**Figure 15, 16**). A shorter heat shock pulse might have removed less stop cassettes. Axons of the ORNs projecting their axons to the DA2 glomeruli could be visualized after acquiring maximum intensity projections. Synaptic boutons could also be resolved (**Figure 15, 16**). They are depicted as green, red, and yellow dots with a higher brightness than the other areas of the DA2 glomeruli, because of the high synaptic activity in these areas (**Figure 15, 16**). However, these areas of presynaptic terminals should have been differentially labeled in order to resolve the interaction between OSNs.

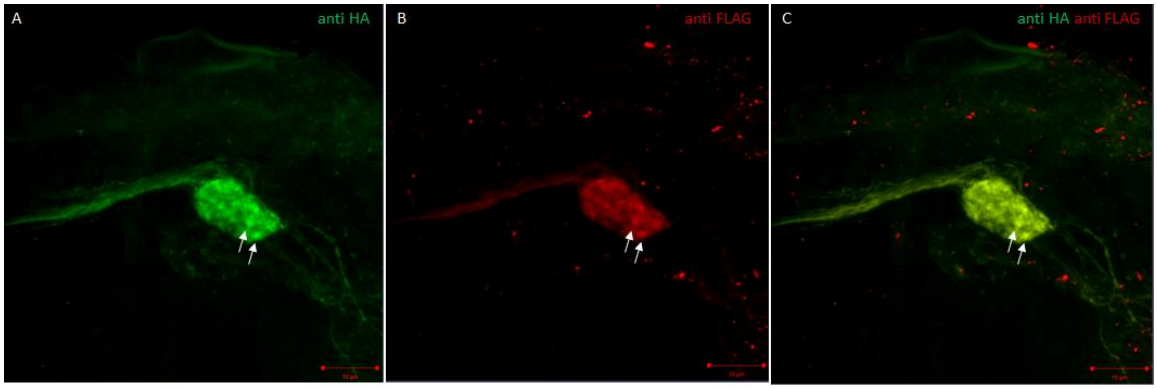

Figure 15. Maximum intensity projections of the right DA2 glomerulus obtained by LSCM 880 with Airyscan of female adult flies dissected three days after being subjected to a heat shock treatment of 15 min. HA and FLAG were co-expressed in the same OSNs of the DA2 after immunohistochemistry (anti-HA, anti-FLAG, anti V5). V5 was not expressed. **(A)** DA2 glomerulus labeled by anti-HA antibody (green). **(B)** DA2 glomerulus labeled by anti-FLAG antibody (red). **(C)** Merged image from **(A)** and **(B)**. Arrows indicate synaptic boutons (green/red/yellow bright dots). Scale bar indicates 10 µm. Magnification (63X).

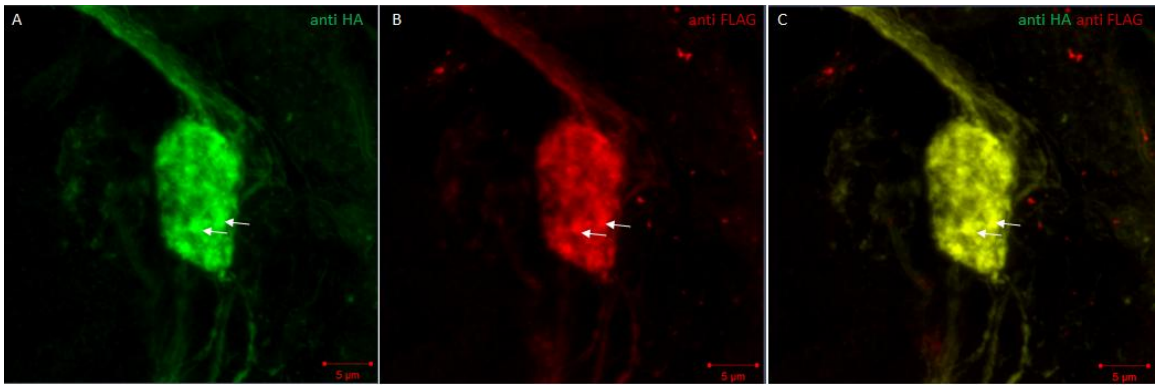

Figure 16. Maximum intensity projections of the right DA2 glomerulus acquired by means of confocal microsopy of female adult flies dissected three days after being subjected to a heat shock treatment of 15 min. HA and FLAG were co-expressed at the same OSNs of the DA2 after immunohistochemistry (anti-HA, anti-FLAG, anti-V5). V5 was not expressed. **(A)** DA2 glomerulus labeled by anti-HA antibody (green). **(B)** DA2 glomerulus labeled by anti-FLAG antibody (red). **(C)** Merged image from **(A)** and **(B)**. Arrows indicate synaptic bouton (green/red/yellow bright dots). Scale bar indicates 5 µm. Magnification (63X).

Even though 63 X glycerol immersion objective (LCI Plan-Neofluar, NA: 1.3, Carl Zeiss) of the LSCM 880 with Airyscan (Carl Zeiss, Jena, Germany) was utilized for the image acquisition, it was impossible to resolve the inner structures of the synaptic boutons (**Figure 15, 16**). An enhanced resolution, a higher magnification, and less co-expression of

the immunoepitope tags in the presynaptic terminals appeared to be crucial for the above-mentioned purpose.

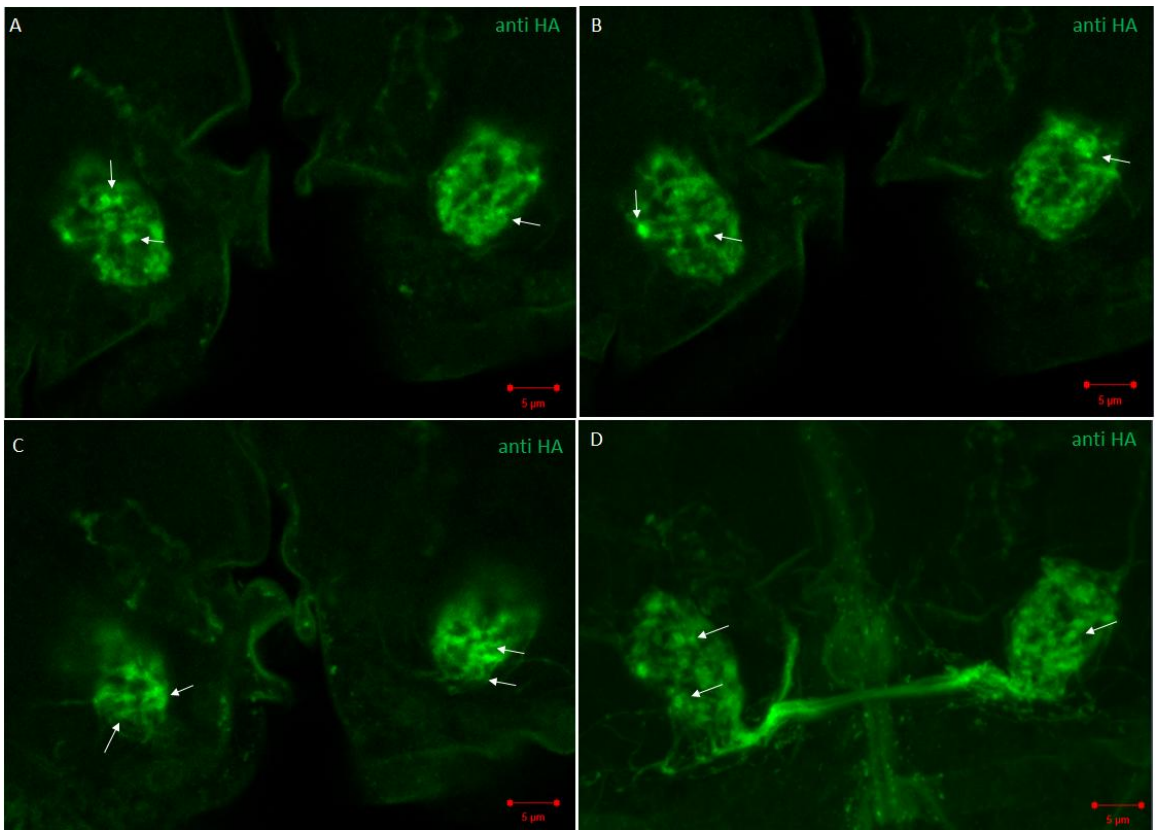

Figure 17. Frontal confocal projections of the DA2 glomeruli in different depths of female adult flies dissected three days after being subjected to a heat shock treatment of 15 min. (A) DA2 glomeruli labeled with anti-HA antibody (green). **(B)** DA2 glomeruli labeled with anti-HA antibody (green) in a different depth. **(C)** DA2 glomeruli labeled with anti-HA antibody (green) in a different depth. **(D)** Maximum intensity projection of the DA2 glomeruli labeled with anti HA-antibody (green). Arrows indicate area of synaptic boutons (green bright dots). Scale bar indicates 5 µm. Magnification (63X).

In pursuance of resolving the inner structures of the synaptic bouton, a higher zoom of the 63 X glycerol immersion objective (LCI Plan-Neofluar, NA: 1.3, Carl Zeiss) was tried. The synaptic boutons could be better visualized (**Figure 16**). However, the areas of presynaptic terminals expressed the same immunoepitope tags, making it impossible to investigate nonsynaptic communication i.e. synaptic spinules in these conditions.

As the resolution appeared to be enhanced by utilizing the goat anti-chicken Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, A11039), frontal confocal projections of the DA2 glomeruli in different depths were obtained (**Figure 17**). The resolution of the

acquired images was further enhanced and the OSNs projecting within the DA2 glomeruli could be easily resolved with a high signal to noise ratio. The ipsilateral and contralateral innervation of OSNs in the DA2 glomeruli was also visible (**Figure 17 D**).

In conclusion, interaction of neighbouring OSNs could not be visualized via immunoprocessing of different expressed immunoepitope tags after a heat shock treatment of 15 min, because HA and FLAG were co-expressed in the areas of presynaptic terminals i.e. synaptic boutons.

3.1.2.5. Heat shock treatment of 8 minutes

The heat shock treatment of 15 min showed that adult flies had to be subjected to a shorter heat shock pulse in order to generate fly brains with differentially labeled neurons or less immunostained neurons within the DA2 glomerulus. The more FRT-stop-FRT sites are flanked by the hs-FLP, the more immunoepitope tags get expressed in the fly brain (Nern, Pfeiffer et al. 2015). A long heat shock generated OSNs within the DA2 glomerulus co-expressing the immunoepitope tags after immunohistochemistry against HA, FLAG, and V5 (**Figure 15, 16**). To resolve nonsynaptic communication via synaptic spinules, less immunoepitope tags co-expression was required, preferably only HA and V5 in different OSNs. They were detected by utilizing the 488 mm and 633 laser lines, hence generating confocal stacks without the possibility of cross-reaction of the emission between the processed immunoepitope tags.

Female adult flies, dissected three days after being subjected to a heat shock pulse of eight min, indeed appeared to express different immunoepitope tags in different OSNs within the DA2 glomerulus (**Figure 18, 19, 20**). The MCFO technique produced a 50:50 ratio of expressed immunoepitope tags. HA and V5 were expressed in 10 flies, while the FLAG could not be detected (**Figure 18, 19**). HA and FLAG were expressed in the other 10 flies, while no signal was detected for the V5 (**Figure 20**).

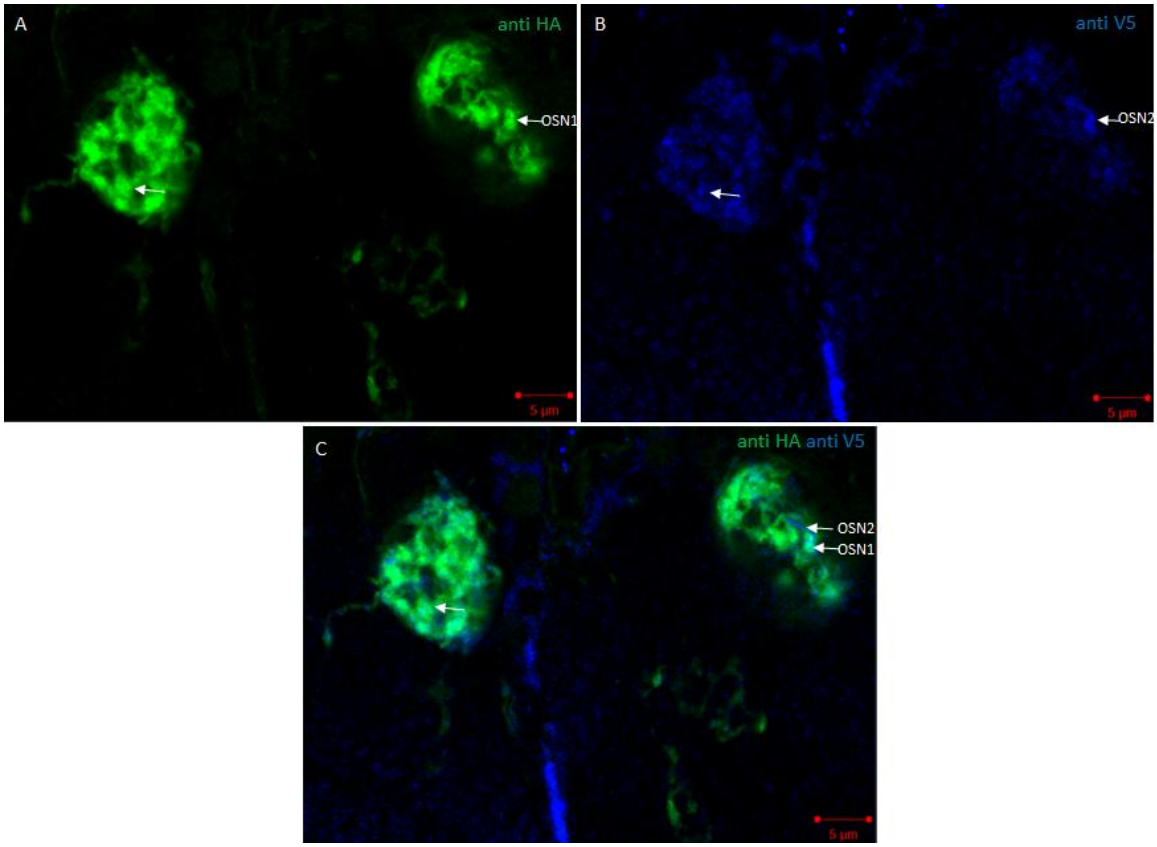

Figure 18. Frontal confocal projections of the DA2 glomeruli of female adult flies dissected three days after being subjected to a heat shock pulse of eight min. (A) DA2 glomeruli labeled with anti-HA antibody (green). **(B)** DA2 glomeruli labeled with anti-V5 antibody (blue). **(C)** Merged image of **(A)** and **(B)**. Arrows indicate differently immunostained neighbouring OSNs, because of the stochastic removal of the FRT-stop-FRT sites. Scale bar indicates 5 µm. Magnification (63X).

HA was expressed in a few OSNs in all of the 20 flies that were used for the experiment. Nevertheless, structures that were immunostained with the HA tag were always easy to be visualized, because the HA tag was always detected with the Argon 488 nm laser (Carl Zeiss, Jena, Germany). The short wavelength of the laser i.e. stronger detection power might have eased the detection of the HA tag compared to the other tags. HA and V5 were expressed in the same cells, but also in different single cells (**Figure 19 C**).

After tracing single OSNs in different depths of the DA2 glomeruli, it appears that the OSNs expressed only one single immunoepitope tag through the whole membrane of the axon (**Figure 18**). The OSNs ought to be in the same plan of a very dense DA2 glomerulus in order to visualize synaptic interactions among them. Afterwards, these OSNs projections were found out to communicate with each other within the DA2 glomerulus. Differentially immunostained OSNs were observed throughout the different depths of the DA2 glomerulus (**Figure 18 C**), confirming the array of multicolored cells following the heat shock treatment of eight min. OSN1 and OSN2 appear to be differentially labeled (**Figure 18 A, B, C**). The interaction between the OSN1 and OSN2 (**Figure 18 C**) might be a membrane invagination of the postsynaptic terminal of the OSN1 to the presynaptic terminal of OSN2.

After merging the confocal stack obtained by using the 488 nm laser line with the confocal stack acquired by using the 633 nm laser line, other invaginations between single-color OSNs were found in different plans of the DA2 glomerulus (**Figure 19 A, B, C**). In a few of the obtained confocal stacks, due to the MCFO technique, differentially immunostained adjacent OSNs, and the enhanced resolution, vesicle-like structures were found within the DA2 glomerulus. These structures are referred to as double membrane vesicles (DMVs) and they are released inside the presynaptic terminals of the OSNs. They are predicted to originate from synaptic spinules (Gruber, Rybak et al. 2018).

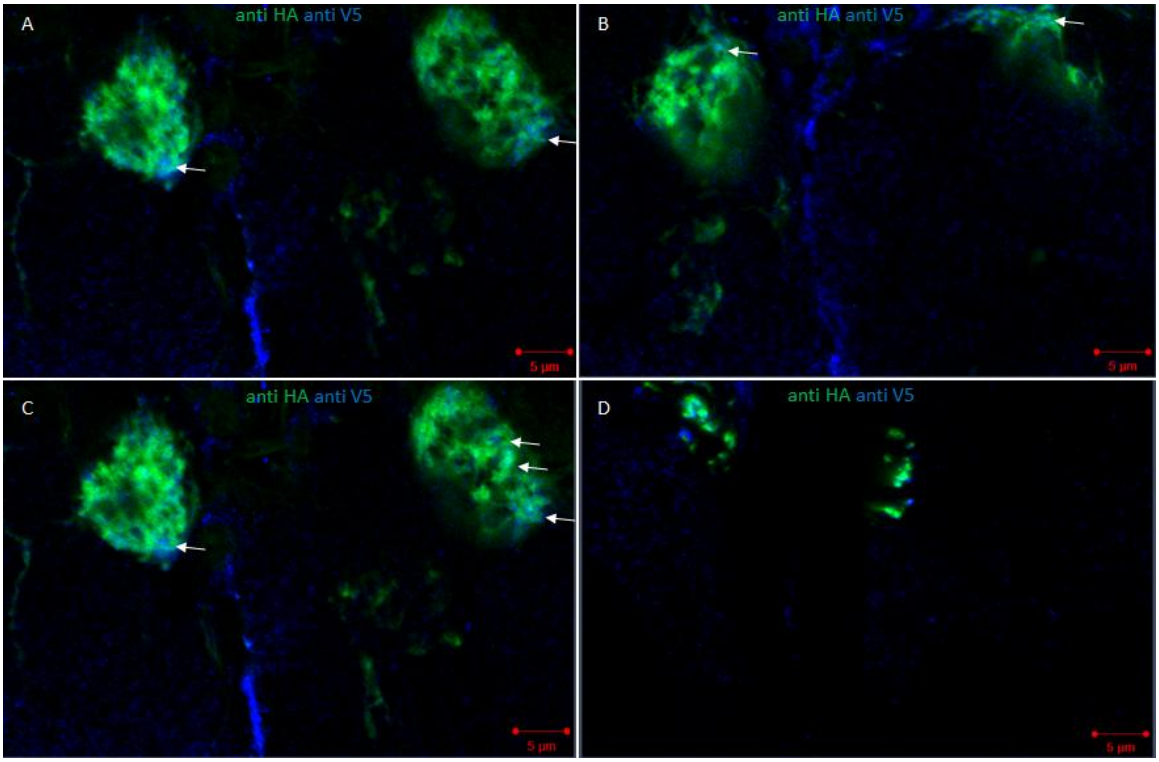

Figure 19. Frontal confocal stacks of the DA2 glomeruli of female adult flies dissected three days after being subjected to a heat shock treatment of eight min. (A) Merged image of the DA2 glomeruli labeled with two antibodies (anti-HA (green) and anti-V5 (blue)). **(B)** Merged image of the OSNs innervating the DA2 glomeruli labeled with two antibodies. **(C)** Merged image of the DA2 glomeruli in a different depth labeled with two antibodies (anti-HA and anti-V5). **(D)** Merged image of differentially labeled OSNs with two antibodies (anti-HA and anti-V5) projecting their axons contralateral to the DA2 glomeruli. Arrows indicate differently immunostained interacting OSNs within the DA2 glomerulus. Scale bar indicates 5 µm. Magnification (63X).

DMV-like structures were detected across the right DA2 glomerulus of fly brains heat shocked for eight min (**Figure 20**). These structures were visible, because they were depicted as HA-labeled vesicles inside FLAG-labeled OSNs and vice-versa. The membrane of distinctive OSNs appears to have been labeled in one single immunoepitope tag. HA and FLAG were both expressed in different colors. However, a few of the immunostained OSNs showed co-expression of the immunoepitope tags (**Figure 20**).

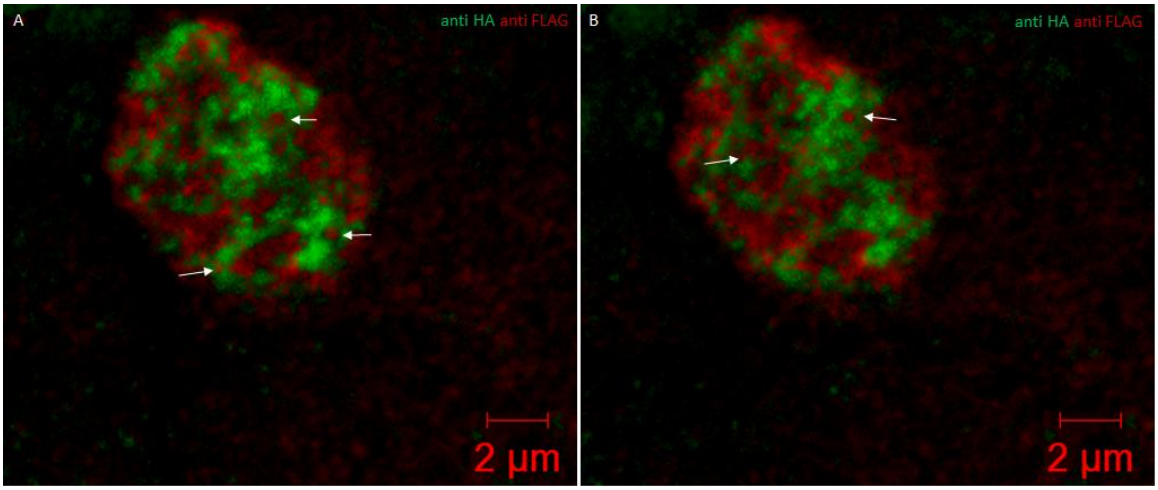

Figure 20. Frontal confocal projections of the right DA2 glomerulus of female adult flies dissected three days after being subjected to a heat shock pulse of eight min. (A) DA2 glomerulus labeled with two antibodies (anti-HA (green) and anti-FLAG (red)). **(B)** DA2 glomerulus in a different depth labeled with two antibodies (anti-HA and anti-FLAG). Arrows indicate DMV-like structures (green and red). Scale bar indicates 2 µm. Magnification (63X).

Despite the burdensome of generating an array of different colored OSNs, it was shown that the heat shock pulse of eight min combined with an immunohistochemistry detecting all the immunoepitope tags could generate confocal stacks, where the neighbouring interacting OSNs could be differentially labeled (**Figure 18, 19, 20**).

3.1.2.6. Heat shock treatment of 5 minutes

A shorter time frame of heat shock pulse was carried out to check if the MCFO could produce a higher signal to noise ratio than the heat shock pulse of eight minutes. Therefore, female adult flies were subjected to a five min heat shock pulse and immunostained with three different antibodies (anti-HA, anti-FLAG, anti-V5).

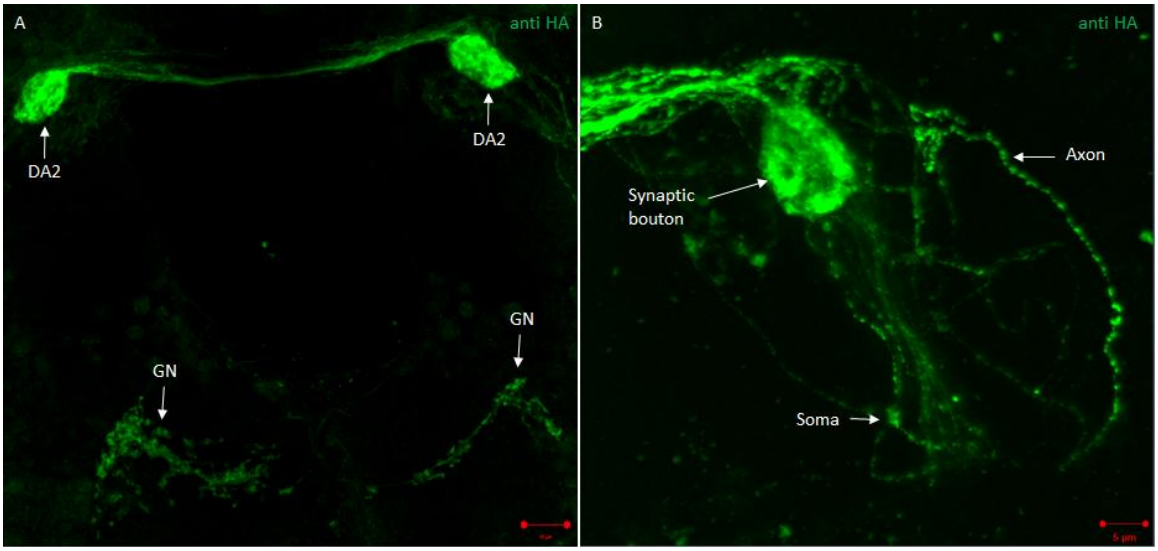

Figure 21. Maximum intensity projections of DA2 glomeruli of female adult flies dissected three days after being subjected to a heat shock pulse of five min. (A) Maximum intensity projection of the DA2 glomeruli immunostained against HA immunoepitope tag. Magnification (40X). Scale bar indicates 10 µm. **(B)** Maximum intensity projection of the left DA2 glomerulus immunostained against HA immunoepitope tag. Scale bar indicates 5 µm. Magnification (63X).

A heat shock pulse of five min showed an expression of the HA immunoepitope tag in all of the OSNs within the DA2 glomerulus. Other immunoepitope tags could not be detected (**Figure 21**). Besides the DA2 glomeruli, other structures below the DA2 glomeruli, such as gustatory neurons (GNs), appeared to be immunostained (**Figure 21 A**). After image acquisition, the signal to noise ratio appeared to be very high. Synaptic boutons could be visualized (**Figure 21 B**) and the axons (**Figure 21 B**) projecting contralateral and ipsilateral could be resolved with the 63 X glycerol immersion objective (LCI Plan-Neofluar, NA: 1.3, Carl Zeiss). Somas, also called cell bodies, were also visible (**Figure 21 B**). Interestingly, a few OSNs located outside of the DA2 glomerulus appear to have been immunostained (**Figure 21 B**).

3.2. OSN-PN experiment

In order to visualize the interaction between afferent OSNs reaching the glomerulus and afferent PNs leaving the glomerulus, a combination of two compatible binary systems of expression was used.

3.2.1. Transgenic flies expressing two binary systems

GAL4/UAS system was used to label the OSNs of the DM2 glomeruli and the QF/QUAS system was used to label the PNs of the GH146-QF enhancer trap line. As it is already investigated, OSNs expressing Or22a establish synapses with PNs, so the latter could project their axons to higher olfactory centers (Jefferis, Potter et al. 2007, Rybak, Talarico et al. 2016). PNs, as OSNs, are shown to receive spinules, but less frequent than OSNs (Gruber, Rybak et al. 2018).

To explore if synaptic spinules between OSNs and PNs could be visible via light microscopy, immunostaining against GFP and mtdTomato was carried out. After image acquisition, DM2 glomeruli appear to have been immunostained with the anti-GFP antibody (**Figure 22 A**), but the same frontal projection detecting the anti-mtdTomato antibody contained only unspecific labeling. PNs were not visible (**Figure 22 B**). Synaptic spinules could not be resolved, because PNs could not be visualized inside the DM2 glomeruli, and neither their axons projecting outside of the DM2 glomeruli (**Figure 22 B**). Different concentrations of the primary and secondary antibodies were applied, but the same results were obtained (**Data not shown**). The DM2 could be visualized after image acquisition of 15 flies dissected five days after eclosion, obtained from the F1 generation of the genetic cross (**see Methods, Figure 10**). However, PNs could not be visualized.

The genetic cross might have not been succeessful, so it was repeated in order to generate more offspring. Nevertheless, the same results were obtained after immunohistochemistry and image acquisition (**Data not shown**). Put together, the construction of transgenic flies expressing both binary systems (GAL4/UAS; QF/QUAS) appeared to be inefficacious.

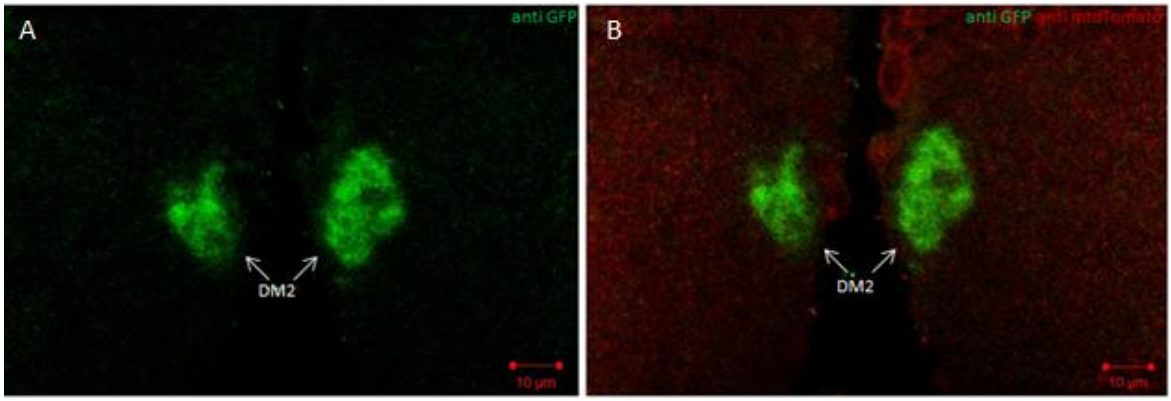

Figure 22. Frontal confocal projections of the DM2 glomeruli of female adult flies dissected five days after eclosion, after immunohistochemistry with two antibodies (anti-GFP and anti-RFP). (A) DM2 glomeruli immunostained with anti-GFP antibody (green). **(B)** Merged image of A and the same frontal projection for the anti-RFP (red). Scale bar indicates 10 µm. Magnification (63X).

3.2.2. Transgenic flies expressing QF/QUAS system

To check if the construction of transgenic flies expressing both binary systems (GAL4/UAS; QF/QUAS) was indeed inefficacious, 10 female adult flies expressing the GH146-QF/QUAS-mtdTomato were dissected five days after eclosion. As the immunohistochemistry of the F1 generation, it appeared that PNs of the GH146-QF enhancer trap line were not labeled with the anti-RFP antibody (**Figure 23 B**). ALs and the glomeruli within the ALs could be visualized after image acquisition, because of the usage of the general neuropil marker (**Figure 23 A**). However, an interaction between the glomeruli and the PNs could not be resolved via light microscopy.

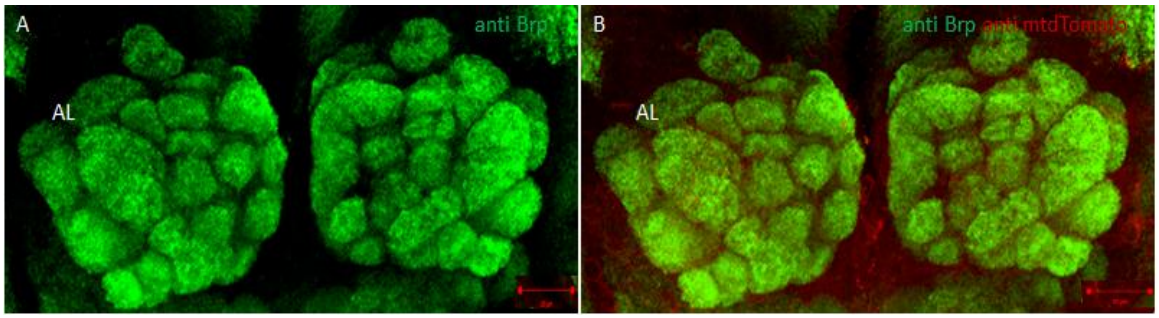

Figure 23. Frontal confocal projections of the ALs of female adult flies dissected five days after eclosion, after immunohistochemistry with two antibodies (anti-Brp and anti-RFP). (A) ALs labeled with anti-Brp (nc82) as a general neuropil marker (green). **(B)** Merged image of A and the same frontal projection for anti-RFP (red). Scale bar indicates 20 µm. Magnification (63X).

The experiment was repeated by using a higher concentration (1:200) of the rabbit anti-RFP antibody to immunostain against mtdTomato. The ALs could be easily visualized (**Figure 24 A, C**). Nevertheless, the same frontal projection detecting the anti-RFP antibody appeared to contain only unspecific labeling. PNs of the GH146-QF enhancer trap line were not immunostained (**Figure 24 B, D**).

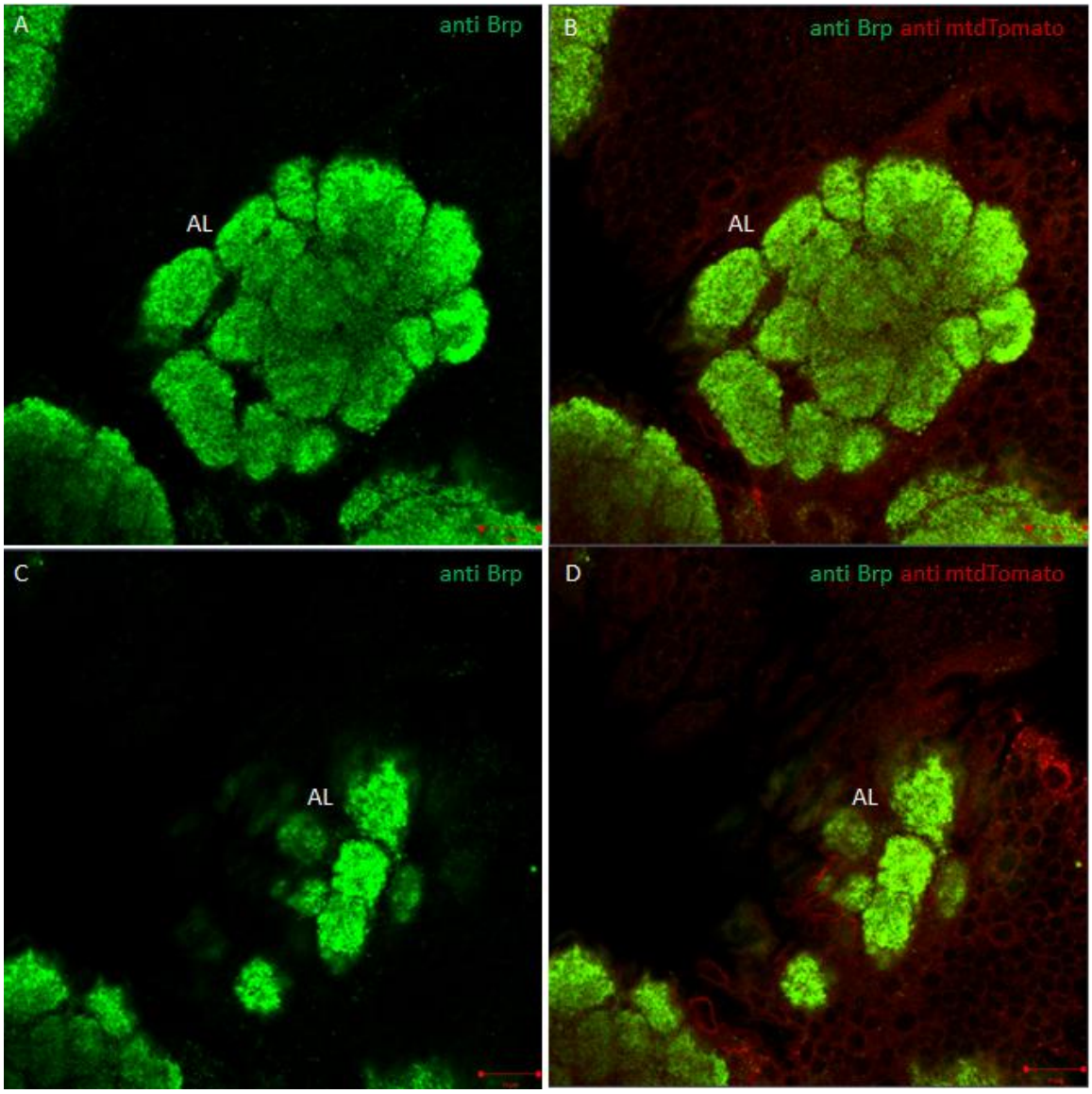

Figure 24. Frontal confocal projections of the right AL of female adult flies dissected five days after eclosion., after immunohistochemistry with two antibodies (anti-Brp, anti-RFP). (A) Right AL labeled with nc82 as a general neuropil marker (green). **(B)** Merged image of **(A)** and the same frontal confocal projection detecting RFP (red). **(C)** Right AL in a different depth labeled with nc82 as a general neuropil marker (green). **(D)** Merged image of **(C)** and the same frontal confocal projection detecting RFP (red). Scale bar indicates 10 μm. Magnification (63X).

4. Discussion

The processing by the chemosensory information has been thoroughly explored by different approaches in the fruit fly. Anatomical, behavioral, and physiological approaches have been carried out to fully understand how the chemosensory information is processed into the AL, as the first olfactory center of the fruit fly (Fiala 2007, Wilson 2013, Gruber, Rybak et al. 2018, Sayin, Boehm et al. 2018). OSNs of the third antennal segment and the maxillary palp expressing the same odor-selective receptor, converge into one of the glomeruli of the AL by establishing synapses with other OSNs, PNs, and LNs (Fiala 2007, Rybak, Talarico et al. 2016). However, it has been revealed that neurons can also communicate through nonsynaptic communication, via invaginating projections (Petralia, Wang et al. 2015). These projections are formed by the invagination of the presynaptic terminal of one neuron and the protrusion of the postsynaptic terminal of another neuron toward the invagination. In mammals, these structures are referred as synaptic spinules (Tarrant and Routtenberg 1977, Petralia, Wang et al. 2015). Evidence of nonsynaptic communication via synaptic spinules was also found in the OSNs expressing Or56a of *D. melanogaster* with the aid of FIB-SEM (Gruber, Rybak et al. 2018).

This study aimed at investigating the nonsynaptic communication via synaptic spinules in the olfactory sensory system of *D. melanogaster* with the aid of high-resolution fluorescence microscopy. For this purpose, differentially labeled interacting OSNs were needed in order to distinguish between the presynaptic terminal of one neuron and the postsynaptic terminal of another neuron by using different fluorophores at each single neuron.

The utility of the MCFO technique was explored as a stochastic labeling technique to differentially label neighbouring OSNs of the DA2 glomerulus. Different timespans of heat shock pulses were carried out in order to establish the optimal timespan for an array of differentially colored neurons in the DA2 glomerulus. Put together, a novel genetic protocol combined with high resolution microscopy was established as a potential technique to further investigate synaptic spinules in the olfactory sensory system of *D. melanogaster*.

4.1. OSN-OSN interaction

The reconstruction of a virtual fly brain has been generated by the usage of single-color labeling (Chiang, Lin et al. 2011). Novel techniques such as Brainbow and Flybow were also developed to stochastically label different populations of neurons and to study neural circuitry, e.g. circuitry of different OSNs expressing the same selective-odor receptor (Hadjieconomou, Rotkopf et al. 2011, Hampel, Chung et al. 2011). Besides Brainbow and Flybow, a new method called MCFO could also be used as a multicolor stochastic technique. Given that the heat shock pulse controls the expression of the hs-FLP, which randomly removes the FRT-stop-FRT cassette, thus expressing the immunoepitope tags, the labeling density could be easily controlled (Nern, Pfeiffer et al. 2015). The MCFO approach has already been established in the visual system and the glia cells of the fruit fly (Nern, Pfeiffer et al. 2015, Kremer, Jung et al. 2017). The immunoepitope tags were detected with a high sensitivity after applying the MCFO technique to the glial cells. The morphology of individual cells at a high resolution and the interaction between different glial subtypes was achieved (Kremer, Jung et al. 2017). The morphology of individual cells and their cellular arrangements were also revealed by means of the MCFO technique with four stop-cassettes (HA, V5, FLAG and OLLAS) in the visual system of *D. melanogaster* (Nern, Pfeiffer et al. 2015).

Nevertheless, the olfactory sensory system of the fruit fly has never been investigated by means of MCFO technique. In this study, a standardization of the MCFO technique was carried out in pursuance of a differentially immunostained DA2 glomerulus in the chemosensory system of the fruit fly. The developmental stage of the fly, the heat shock timespan, the genetics i.e. constructions of transgenic flies, the image acquisition, and the immunohistochemistry were the most crucial variables that needed to be optimized.

4.1.1. The appropriate developmental stage of the fly

To decide the appropriate developmental stage of the fly that would generate differently immunostained OSNs within the DA2 glomerulus and the best reporter expression with the highest resolution, a heat shock pulse of 20 min was introduced to pupae and larvae of the same vial of the F1 generation (**See Methods, Figure 9**). Further, a heat shock pulse of 20 min was also introduced to adult flies of the same vial. Following dissection, immunohistochemistry, and image acquisition, no signal could be detected from the brains of larvae and pupae (**Figure 12**). Quite the contrary was observed after image acquisition of the brains of adult flies. As all of the immunoepitope tags were expressed, hs-FLP appeared to have removed all the FRT-stop-FRT cassettes. This generated a co-expression of HA, FLAG, and V5 immunoepitope tags in the OSNs of the DA2 glomerulus (**Figure 13**).

Even though OSNs start to project axons to the DA2 glomerulus early at development of the fly, the Or56a may not be expressed yet during this developmental stage. An Or56a-GAL4/UAS-immunoepitope tags was inserted into the flies during the genetic cross. This would imply the transcriptional driver GAL4 not being expressed, hence the immunoepitope tags not being translated either. Put together, the GAL4 and the immunoepitope tags might have not been expressed at that point of developmental stage of the flies, thus restraining the accumulation of the immunoepitope tags in the OSNs expressing the Or56a in the sufficient amount that could have been followed by fluorescence microscopy visualization. In conclusion, the aforementioned reasons could have made the heat shock pulse to larvae and pupae unsuccessful to generating a multicolored array of OSNs.

4.1.2. Heat shock is crucial for the expression of the tags

To check if the heat shock treatment induced the expression of the immunoepitope tags in the OSNs of the DA2 glomerulus, flies were dissected immediately after eclosion without being subjected to a heat shock treatment. After image acquisition, no signal could be detected at the DA2 glomerulus, suggesting that the heat shock treatment is crucial for the inducement of the hs-FLP and the transcription of the immunoepitope tags, thus the visualization of the OSNs via fluorescence microscopy after immunoprocessing of the immunoepitope tags (**Figure 14**).

Furthermore, it has been suggested that rearing the flies at at 25 ^{0}C might cause a leakiness of the MCFO system and that flies should be reared at 18 ^{0}C (Au - Batelli, Au - Kremer et al. 2017). To check if the hs-FLP was also active during 25 ^{0}C instead of 37 ^{0}C by generating a leakiness of the system, female adult flies were dissected immediately after eclosion and five days after eclosion without being subjected to a heat shock treatment. This experiment was executed to check if the hs-FLP would remove some FRT-stop-FRT cassettes after five days, hence expressing few immunoepitope tags. No signal was detected after image acquisition of the brains of flies dissected immediately after eclosion (**Figure 14**). Same results were obtained from the flies dissected five days later (**Data not shown**). OSNs of the DA2 glomerulus appeared to be unlabeled, hence the hs-FLP could not be induced during 25 ^{0}C. In this study, only 37 ^{0}C could generate differentially labeled OSNs in the olfactory sensory system of the fruit fly. To achieve a sparse labeling and an enhanced resolution, all the other experiments consisted on flies that were heat shocked at a temperature of 37 ^{0}C.

4.1.3. Optimal heat shock timespan

Following the validation of the heat shock treatment as crucial for the expression of the immunoepitope tags in the DA2 glomerulus of adult flies, the appropriate timespan of heat shock appeared to be pivotal to further investigating synaptic spinules within the DA2 glomerulus. Different timespans such as 20 min, 15 min, 8 min, and 5 min were tried. The goal was to induce the expression of the immunoepitope tags without co-expression between adjacent OSNs, or to generate just a few immunostained OSNs. A less dense DA2 would imply an enhanced resolution of the confocal stacks.

A timespan of 20 min appeared to induce a co-expression of the three immunoepitope tags (HA, FLAG, V5) in the OSNs of the DA2 glomerulus (**Figure 13**). A co-expression of two immunoepitope tags (HA, FLAG) was detected after a heat shock treatment of 15 min. V5 was not expressed (**Figure 15, 16**). Only one expressed immunoepitope tag (HA) was observed after a heat shock treatment of five min (**Figure 21**). Furthermore, a heat shock treatment of eight min was carried out to 20 female adult flies. Following immunohistochemistry and image acquisition, 10 flies appeared to have only HA and V5 immunoepitope tags expressed in different OSNs. Co-expression of the immunoepitope tags was also observed (**Figure 18, 19**). In the other 10 flies, an expression of HA and

FLAG immunoepitope tags was observed after image acquisition in different OSNs. Co-expression was also overved (**Figure 20**).

Even though the synaptic spinules could not be resolved in the female adult flies after a heat shock pulse of 15 min, this experiment revealed an important aspect of the MCFO technique. The smGFP carrying the immunoepitope tags HA, FLAG, and V5 is designed in such a way to be bound to the plasma membrane via a N-terminal myristoylation signal (Pfeiffer, Ngo et al. 2010, Nern, Pfeiffer et al. 2015). This generates a sparse labeling of the dendrites and axons. Frontal confocal projections of the DA2 glomeruli appeared to have a high signal to noise ratio (**Figure 17**). Furthermore, axons and synaptic boutons could be clearly visualized. Confocal stacks obtained by utilizing the 488 nm laser line showed an enhanced resolution after detection of the HA immunoepitope tag. Axons and synaptic boutons could also be clearly visualized after a heat shock treatment of five minutes (**Figure 21**).

In (**Figure 17, 21**) neurons located on the outter part of the DA2 glomeruli were observed. They might express Or56a, but they were not observed after immunohistochemistry of flies expressing Or56a-GAL4/UAS-GFP (**Data not shown**). These particular neurons might express the Or56a, explaining why they were immunostained by the MCFO method, or they might be part of other glomeruli and unspecific immunostaining might have occurred. However, the immunostained OSNs on the outer part of the DA2 glomeruli were observed in all the experiments.

Furthermore, the gustatory projecting neurons appeared to be immunostained after a heat shock pulse of five minutes (**Figure 21 A**). A comparative approach between flies that have been genetically labeled with the GAL4/UAS binary system and the MCFO technique is needed to further investigate the observed phenomena.

4.1.4. Synaptic spinules and DMVs

The MCFO technique was introduced to the OSNs of the DA2 glomerulus to generate a differentially genetic labeling of adjacent OSNs (Nern, Pfeiffer et al. 2015). A heat shock treatment of eight minutes was observed to be the optimal timespan to investigate synaptic spinules, because differentially immunostained neighbouring OSNs were generated (**Figure 18, 19, 20**). OSN1 appears to have the HA tag expressed (**Figure 18 A**), while OSN2 appears to have the V5-tag expressed (**Figure 18 B**). The filters used to detect these tags do not overlap, hence cross-talk between the different fluorescence emissions was not possible. In (**Figure 18 C**) OSN1 and OSN2 appeared to be interacting. One of the structures appears to be invaginating toward the other one (**Figure 18 C**). By comparing the above-mentioned finding with the image obtained with the aid of FIB-SEM (**Figure 7 A**), the protrusion formed by OSN1 towards OSN2 might be a synaptic spinule. However, a better resolution is crucial to resolve the synaptic spinules and to validate that this finding is indeed a synaptic spinule.

Following the invagination of the postsynaptic OSNs into the presynaptic OSNs, pinched-off materials referred to as DMVs are released inside the presynaptic terminal (Gruber, Rybak et al. 2018). This has been revealed with the aid of FIB-SEM, but there is no proof via light microscopy that DMVs exist in the olfactory sensory system of *D. melanogaster*. Classic single-color labeling cannot give this kind of information, because the invagination of the membrane of the postsynaptic terminal of one OSN inside the presynaptic terminal of another OSN should be revealed. By using the GAL4-OR56a, all the OSNs expressing the Or56a that project to the DA2 glomeruli will be immunostained by the same manner, given that GAL4-Or56a fly gets crossed to a UAS-GFP fly. Differentially immunostained adjacent OSNs would be mandatory for the above-mentioned purpose.

DMV-like structures were observed after image acquisition of immunostained brains of female adult flies dissected three days after being subjected to a heat shock pulse of eight min (**Figure 20**). Differentially labeled neighbouring OSNs were generated after a heat shock treatment of eight minutes, thus creating the most appropriate conditions to resolve synaptic spinules or DMVs. To further ensure that the DMV-like structures found on the DA2 glomerulus after a heat shock pulse of eight minutes are the same structures observed by FIB-SEM, super-resolution microscopy such as STED or SIM are needed (Bates, Jones et al. 2013, Gruber, Rybak et al. 2018, Vicidomini, Bianchini et al. 2018).

4.2. OSN-PN interaction

Another approach to investigate synaptic spinules was carried out. Instead of using a multicolor stochastic labeling technique, a technique that combines two different binary systems was used. GAL4/UAS was used to label OSNs of the DM2 glomeruli, while QF/QUAS was used to label the PNs of the GH146-QF enhancer trap line. The latter are observed to establish synapses with neurons inside the ALs (Lai, Awasaki et al. 2008, Rybak, Talarico et al. 2016). Different antibodies were used to immunostain against GFP and mtdTomato, but mtdtTomato could not be detected (**Figure 22**). Therefore, synaptic spinules could not be visualized.

To assess if the QF/QUAS system was not expressed in the crossed flies (**See Methods, Figure 10**), or simply the antibody was problematic, flies expressing only the QF/QUAS system were dissected and immunostained against mtdTomato. Same results were obtained. PNs could not be detected (**Figure 23, 24**). The experiment was repeated several times with different anti-RFP antibodies. However, PNs remained undetected. OSNs of the DM2 glomeruli labeled with the Or22a-GAL4/UAS-GFP binary system were always detected with a high signal to noise ratio. Nevertheless, single-color labeling of OSNs appeared to be insufficient to resolve structures such as synaptic spinules.

4.3. Outlook

A combination of the MCFO technique with Expansion Microscopy (ExM) has been suggested as an opportunity to achieve a better resolution and to facilitate tracing (Chen, Tillberg et al. 2015, Freifeld, Odstrcil et al. 2017). Expansion Microscopy (ExM) is a novel technique that allows physical magnification instead of optical magnification by physically expanding a swellable polymer network containing the sample (Chen, Tillberg et al. 2015). If the immunoepitope tags inserted into the backbone of the sfGFP could be anchored to the swellable gel by applying a modified version of ExM called Protein-Retention Expansion Microscopy (ProExM) a validation via light microscopy of the synaptic spinules in the DA2 glomerulus of the fruit fly could be possible (Pédelacq, Cabantous et al. 2005, Nern, Pfeiffer et al. 2015, Tillberg, Chen et al. 2016, Gruber, Rybak et al. 2018). Furthermore, Protein-Retention Expansion Microscopy (ProExM) has already been introduced to *D. melanogaster* with successful results to reveal the three-dimensional organization of the synaptonemal complex, to investigate the transmembrane protein LPR4, and to analyze fine intracellular structures (Cahoon, Yu et al. 2017, Mosca, Luginbuhl et al. 2017, Jiang, Kim et al. 2018).

Put together, to further investigate synaptic spinules and to generate a valid proof that DMVs indeed originate from synaptic spinules, a combination of the MCFO technique and ProExM could be carried out (Nern, Pfeiffer et al. 2015, Tillberg, Chen et al. 2016, Gruber, Rybak et al. 2018). This would produce a proof of synaptic spinules via light microscopy and it might also turn out to be a technique that allows their quantification. If the quantification of synaptic would be possible by the above-mentioned combination, a comparative morphological approach between DA2 and DM2, respectively involved in aversive and attractive behavior of the fruit fly, could further be investigated. This could lead to functional analysis of synaptic spinules in *D. melanogaster*.

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Declaration of Authorship

I, Geri Braho, hereby declare that I am the sole author of this thesis and that it is based on my own work. All direct and indirect sources of information used are cited in the text and acknowledged as references in the bibliography. This thesis has never been submitted for examination elsewhere nor has it been published in German or any other language.

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(Place, Date) (Signature)