An optimized vector for the functional expression of insect odorant receptors

BACHELOR THESIS

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Contents

Glossary

bp	Base pairs
kb	kilo bases
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
RNA	Ribonucleic acid
dNTPs	Deoxyribonucleotides
PCR	Polymerase chain reaction
µl	Micro liter
mM	Millimolar
nM	Nanomolar
rpm	Revolutions per minute
RCF	Relative Centrifugal Field
ml	Milliliter
g	Gram
ng	Nanogram
pmol	Picomol
min	Minute
s	Second
U	Unified atomic mass unit
LB	Lysogeny broth
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite Repression
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris base acetic acid EDTA
Orco	Odorant receptor co-receptor

IgG Immunoglobulin G

OSN Olfactory Sensory Neuron

OR Odorant Receptor

IR Ionotropic Receptor

GR Gustatory Receptor

GPCR G-protein Coupled Receptors

iGluR Ionotropic Glutamate Receptors

OBP Odorant Binding Protein

SSR Sensillum Electrophysiological Recordings

VUAA1 N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl) thio) acetamide

ER Endoplasmic reticulum

List of Figures

List of Tables

1. Introduction

Food is important to all living species as is it essential for the survival, moreover adaptation over many generations has helped several species to develop a strategy to determine eatable substances. In insects especially, the need to identify nutritional food sources and evaluating whether to consume them is crucial. The fact that there are odors from a high number of sources in the environment, insects have evolved to be very efficient in recognition of these information[1].

1.1. Olfactory system of *Drosophila melanogaster*

Animals receive key information over the sensory systems from the environment ensuring their survival[2]. *Drosophila melanogaster* is a multicellular organism whose olfactory system has similarities to the human olfactory system, they also have several properties making them one of the best model organism in research. Characteristics like their short life span in addition to their easy diet and high reproduction, also contribute to make *Drosophila* the best model organism of choice[3].

As hinted above, *Drosophila melanogaster*, also know as, vinegar fly, has a well-developed olfactory sensory system which allows them to locate a great variety of potential food sources, mating partners, as well as to avoid dangerous predators or pathogenes[4, 5, 1]. The fly possesses two major olfactory organs, the antennae and the maxillary palps located on the fly's head (Fig 1)[6]. The third segment houses hair-like structures called sensilla. Depending on the size and shape of these structures they are differentiated into basiconic, intermediate, trichoid and coeloconic. Every sensillum encases dendrites of up to four different sensory neurons housing the chemosensory receptors. Gustatory receptors (GR) are responsible for the Carbon-dioxide (CO_2) detection, and also recognize bitter and sweet testants[1]. They are seven transmembrane domain proteins that work in dimers, like in the case of CO_2 detection shown in figure 2.

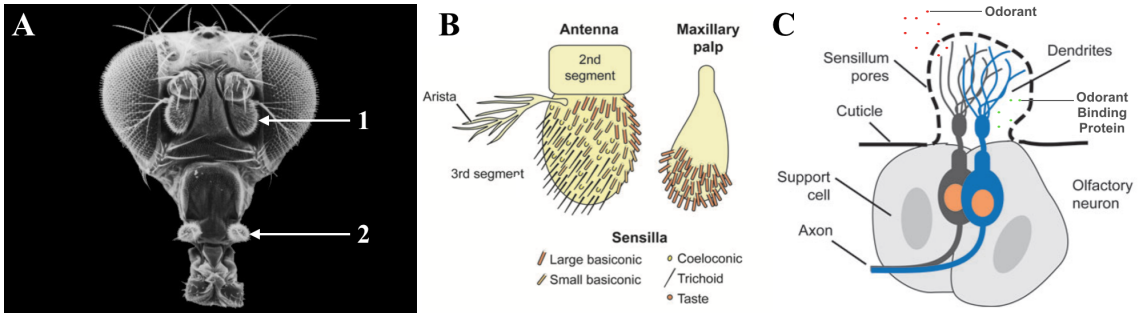

Figure 1.: A *Drosophila melanogaster* head with its antennae (1) and maxillary palps (2) modified from [7] **B** Representation of the antennae, the maxillary palp and the different sensilla, modified from [8] **C** Representation of one sensillum with two neurons, modified from [8].

Ionotropic receptors (IR) are postulated to have developed from ionotropic glutamate receptors (iGluRs) and sense acids and amines[1]. Glutamate receptors appear in most animals and have, like iGluRs (see Fig. 2) an extracellular domain responsible for ligand binding[9]. The third type of receptor called odorant receptor (OR) can detect odorants in general and also pheromones.

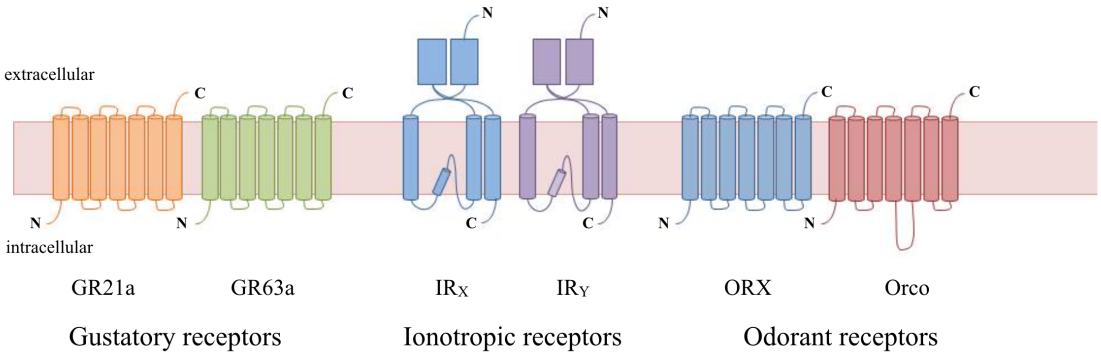

Figure 2.: Representation of the different receptor types.
Gustatory receptors (GRs) here shown: GR21a and GR63a responsible for CO_2 detection [4], Ionotropic receptors (IRs) consisting of a specific receptor IR_X and a co-receptor IR and odorant receptors (ORs) with the specific receptor (ORX) and a common co-receptor $(Orco)_Y$[9].

ORs are heteromeres composed of a specific receptor and an odorant co-receptor both consisting of a seven-transmembrane domain with a intracellular N-terminus and an extracellular C-terminus (Fig. 2). This structure is an inverted version of G-protein coupled receptors despite having little to no sequence similarity to it[10, 1]. The odorant co-receptor (Orco) is ubiquitous being present in all ORX expressing OSNs. Orco has been shown to be required for the ORs' correct localization to the dendritic membrane of the neuron[11, 6]. In contrast the variable ligand specific odorant receptor (ORX) determines the ligand binding and forms an ion pore

together with Orco[1].

When an odorant reaches the sensillum, it passes through pores of the outer cuticle. The inside is filled with sensillum lymph containing odorant binding proteins (OBPs) which are released from adjoined support cells. The OBPs bind odorant molecules and supposedly help them find their way to the neuronal dendrites where upon binding and activating the ORX and Orco complex an action potential is triggered. The signal is forwarded to the brain via axons[10].

1.2. State-of-the art methods to study odorant receptors

Different techniques have been established in order to evaluate the functional properties of ORs, as well as finding potential ligands. The ectopic expression of a given OR in an otherwise empty *Drosophila* OSN is one way to do so (i.e. the "empty neuron" system). A transgenic fly carrying a given OR is created by molecular and genetic engineering methods, and crossed with other transgene flies to express the given gene in the "empty neuron"[12]. Analysis of the expressed OR for potential ligands is done via single sensillum electrophysiological recordings (SSR). This so-called "empy neuron system" builds a fundamental way to analyze OR functional properties *in vivo*[1]. On the other hand, ORs can also be expressed in heterospecific cell systems. *Xenopus* oocytes are well used for OR characterization. In this case, the ribonucleic acid (RNA) coding for an OR is injected into the egg. The RNA of the odorant co-receptor Orco is required as well in order for the ORX/Orco complex to function in the frog eggs. Testing is done by patch clamping cells, which are stimulated electrically with relevant odor ligands. Previous attempts to express ORs in Sf9 (*Spodoptera frugiperda* ovary) cells showed trafficking problems of the proteins to the plasma membrane[15].

Another way to investigate the functional properties of odorant receptors is by heterologous expression in mammalian cells. HEK293 (Human Embryonic Kidney) cells are commonly used for this purpose. HEK293 is a stabilized cell line used as an expression tool for proteins in general, particularly receptors and ion channels[13]. The advantages of this system are based on its simple and fast reproduction while carrying properties to behave like a neuronal cell, making it a good expression system[14].

Eukaryotic cells can be transiently or stably transfected with DNA plasmid vec-

tors. Once the plasmid has been transfected into the cells and they express the genes of interest, they can be used for functional studies[1]. Stable clones are best for a high and consistent level of expression of the gene(s) of interest, reducing the variability of results[36]. However, the generation of such cell lines is laborious and time consuming. In contrast, a transient expression is faster to generate but produces low levels of expression. Moreover, the transient co-expression of two genes of interest - each one carried in a single plasmid - renders a low number of cells co-expressing both genes, and in low amounts. Increasing the expression by using only one vector, carrying two necessary genes allows transient transfection of good quality and no need to generate a stable cell line. The heterologous expression of ORs in eukaryotic cells is the most relevant technique for this project and formed the basis of my bachelor thesis.

The function of *Drosophila melanogaster* ORs expressed in HEK293 cells can be studied by Ca^{2+} imaging. Calcium imaging is a widely used technique in physiology, whose starting point were the papers published by Sidney Ringer describing the importance of Ca^{2+} for a frogs heart[16, 17, 18, 19]. Due to its importance for many cellular processes especially in neuronal cells, Ca^{2+} became an important indicator for signaling pathways and cell functioning[19, 20, 21, 18]. The principle of this method relies on the change of conformation of fluorophores after binding Calcium-ions what results in a differing wavelength emission[18, 17, 19]. Cells are first filled with fluorescent indicators, e.g. Fura-2, through different techniques including diffusion[17]. In the case of Fura-2, the conformational change when binding Ca^{2+} shifts the wavelength of emission into the visible light range[20]. Once the eukaryotic cells are filled with Calcium-ion indicators, the odorant recepor is activated by exposing the cells to a ligand. This binding of the ligand leads to opening of the channel pore and an influx of Calcium-ions into the cells . The binding of the indicator shifts its fluorescence, what can be detected via fluorescent microscopy.

1.3. Aim of this thesis

The aim of this project was the optimization of a constructed vector to improve the transient transfection and expression of ORs in HEK293 cells, suitable to high-throughput studies. To do so, a suitable plasmid able to carry the two desired genes was the first task, to increase the expression rates and therefore enable consistent expression and results while using transient expression. The humanization of the OR sequence should solve the problems of cross-species transfection.

2. Material

2.1. Chemicals

Substance	Art no.	Company
NaCl	9265.1	Roth, Karlsruhe, Germany
TRIS	2449.2	Roth
Acetic acid	7332.2	Roth
Tryptone \Peptone ex casein	8952.2	Roth
Yeast Extract	2363.3	Roth
Agar-Agar, Kobe I	5210.2	Roth
Glycerol	M92.1	Roth
Ampicillin	K029.2	Roth
D-(+)-Glucose	1724615	Sigma Aldrich, Steinheim,Germany
SOB medium	AE27.1	Roth
Water for chromatography	1153332500	Merck KGaA, Darmstadt,Germany
Ethidium bromide 1 %	2218.2	Roth

2.2. DNA Ladder

Substance	Art no.	Company
2-Log DNA Ladder	N3200S	New England BioLabs, Ipswich, MA
Gel Loading Dye, Purple (6 x)	B7024S	New England BioLabs

2.3. Media, solutions

	Composition
LB medium (1 l)	10 g Tryptone 5 g Yeast Extract 5 g Sodium chloride 1 l Double distilled water pH 7.5
LB agar (200 ml)	200 ml LB medium 3 g Agar 400 µl Ampicillin (50 mg/ml)
1000 x Ampicillin	1 g Ampicillin 100 ml Double distilled water
SOC medium	0.36 g glucose 100 ml SOB medium
50 x TAE-buffer	2 M Tris 2 M Acetic acid 50 mM EDTA Double distilled water 1 l

2.4. Software

Software	**Version**	**Company**
Geneious	6.0.5	Biomatters, Aarhus N, Denmark
Adobe Illustrator	CS5	Adobe Systems, CA

2.5. Enzymes, Kits, plasmids

Substance	Art no.	Company
E.Z.N.A. Plasmid DNA mini Kit I	D6942-02	Omega-Bio-Tek, Norcross, GA
NucleoBond® Xtra Midi EF	740420.10	MACHEREY-NAGEL, Düren, Germany
E.Z.N.A. Gel Extraction Kit	D2501-01	Omega-Bio-Tek
Phusion DNA Polymerase	F530-L	New England BioLabs, Ipswich, MA
Advantage 2 PCR Kit	639206	Takara Bio Europe, Saint-Germain-en-Laye, France
Taq Polymerase	PEQL01-1020	Peqlab Biotechnologie, Erlangen,Germany
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	New England BioLabs
Cutsmart-buffer	B7204S	New England BioLabs
BamHI-HF	R0136S	New England BioLabs
HindIII-HF	R3104S	New England BioLabs
XbaI	R0145S	New England BioLabs
EcoRI-HF	R3101S	New England BioLabs
NaeI	R0190S	New England BioLabs
XhoI	R0146S	New England BioLabs
α Select Gold Efficiency Competent Cells	BIO-85027	Bioline, Luckenwalde, Germany
pBI-CMV1 Vector	631630	Takara Bio Europe, Saint-Germain-en-Laye, France
pCMVTNT	L5620	Promega, Mannheim, Germany
T4 DNA Ligase (1 U/µL)	15224017	Applied Biosystems, Thermo Fisher Scientific, Foster City, CA

2.6. Primers

Primer	Sequence 5'-3'
pCMVTNT-Fwd	CGGTTATCCAGATCGCCCTTCCCCAACAG
pCMVTNT-Rev	CCTGATAAATATAAATGTACATATTATGATA-TAGATACAACGTATGC
pBI-CMV1-Fwd	GTACATTTATATTTATCAGGGTTATTGTCT-CATG
pBI-CMV1-Rev	AAGGGCGATCTGGATAACCGTATTACCG
pBI-Orco-Fwd	GTGTACGGTGGGAGGTCTATATAAG
pBI-Orco-Rev	GTGGTATGGCTGATTATGATCCTCT
hOrcoExon1-Fwd	TTAGTGAACCGTCAGATCCGCTAGGGATC-CGCCACCATGGAACAGAAACTGATCTCT-GAAGAAGACCTGGCTAGCACAACTAGCAT-GCAAC
hOrcoExon1-Rev	CCTTGATACTTACCTGGGCCAGAGCAT-AGCC
hOrcoExon2-Fwd	TTTCTCTCCACAGGTGTTTCACTTTTGCAT-TTTC
hOrcoExon2-Rev	TGATCCTCTGGAGATATCGTCGTCGAC-AAGCTTTCACTTCAGTTGGACCAG
chimeric_intron-Fwd	TGGCTCTGGCCCAGGTAAGTATCAAGG-TTACAAGACAGG
chimeric_intron-Rev	TGCAAAAGTGAAACACCTGTGGAGAGA-AAGGCAAAG
BI_MTSV5ERShOr47a-Fwd	AACCGTCAGATCGCCTGGAGGCCGCC-ACCATGGATCAA
BI_MTSV5ERShOr47a-Rev	CCCGCGGCATATGACCGGTGTCAAGAA-AAGCTGCGCAGC
ChimIntr_Or47a-Fwd	CAGATCTCCTGAGGCTGCAGCACAACAGT-CTCGAACTTAAG
ChimIntr_Or47a-Rev	CCATGGTGGCGGCAAAAAGAACAAGTAGC
CI-MTSV5ERShOr47a-Fwd	TCTTTTTGCCGCCACCATGGATCAAGTC
CI-MTSV5ERShOr47a-Rev	GGTATGGCTGATTATGATCCACTCAAGA-AAAGCTGCGCAG

2.7. Laboratory equipment

Equipment	Type	Company
Electrophoresis system	Mupid One	Biozym Scientific, Wien, Austria
Centrifuge	5810R	Eppendorf, Hamburg, Germany
Centrifuge	5430R	Eppendorf, Hamburg, Germany
Vortex mixer	Vortex-Genie 2	Scientific Industries, Bohemia, NY
Thermocycler	Gene Amp PCR System 9700	Applied Biosystems, Thermo Fisher Scientific, Foster City, CA
Incubator	Heraeus B12	Kendro Laboratory products, Hanau, Germany
Orbital shaker	Novotron A182K	Infors AG, Bottmingen, Switzerland
Agarose gel imaging system	Biovision Superbright mode II 3000	Peqlab Biotechnologie, Erlangen, Germany
Scale	BP 2100S	Sartorius, Göttingen, Germany
Scale	PB3002-S	Delta Range
Photometer	Nanodrop One	Applied Biosystems, Thermo Fisher Scientific, Foster City, CA
Thermomixer	5355 R	Eppendorf, Hamburg, Germany
Pipettes	Pipetman Classic	Gilson, Middleton, WI
Autoclave	2540EL	Systec, Linden, Germany

3. Methods

3.1. Theory of methods

3.1.1. Polymerase chain reaction

Polymerase chain reaction allows amplifying a specific Deoxyribonucleic acid (DNA) target region. The gene specific primers are designed (with e.g. *Geneious* software) and synthesized (e.g. by Eurofins MWG Operon (Ebersberg)). Three steps are performed in which the double strand of DNA is denaturated by increasing the temperature, followed by the annealing of the primers and two new strands of DNA synthesize in the extension step via a polymerase enzyme[22]. The annealing of the primer to the corresponding DNA region requires a specific temperature which depends on the length and composition of the primer itself. Different taq polymerase enzymes elongate the single strands at specific paces, therefore specific time spans, depending on the length of the fragment amplified and the enzyme, are necessary. A high amount of copies is reached through repeating the steps up to 35 times in cycles. There are many polymerase enzymes available from which two were selected, in this work, for different purposes.

The *Phusion* taq polymerase was chosen due to its proof reading characteristics. For this reaction 10 ng of template DNA are used, with a corresponding buffer and dNTPs (table 1). A general protocol in table 2 was followed and shown, time spans and temperatures were adjusted accordingly.

PCR technique was also used to quickly verify the intermediate product of a cloning (see section 5.2.). Here, a regular taq polymerase without a proof reading characteristic (e.g. from *VWR life science*) was chosen. This taq polymerase requires different conditions shown in table 4 and utilizes its own buffer and (table 3).

Table 1.: General composition of a polymerase chain reaction using *Phusion* taq polymerase.

	Volume [µl]
Water	32.5
DNA	1
Phusion Buffer (×5)	10
Forward primer	2.5
Reverse primer	2.5
dNTPs (10 mM)	1
Phusion taq polymerase	0.5
Total	50

Table 2.: General conditions of a PCR using *Phusion* taq polymerase.

Denaturation	30 s	98 °C	
Denaturation	10 s	98 °C	×35 cycles
Annealing	30 s	X °C	
Extension	[30 s per kb]	72 °C	
Final extension	5 min	72 °C	

Table 3.: General composition of a polymerase chain reaction using *VWR life science* taq polymerase.

	Volume [µl]
Water	17.75
DNA	2
Buffer (×10)	2.5
Forward primer	1
Reverse primer	1
dNTP (10 mM)	0.5
VWR life science taq polymerase	0.25
Total	25

Table 4.: General conditions of a PCR using *VWR life science* taq polymerase.

Denaturation	2 min	94 °C	
Denaturation	30 s	94 °C	×35 cycles
Annealing	30 s	X °C	
Extension	[1 min per kb]	72 °C	
Final extension	5 min	72 °C	

Table 5.: General composition of a restriction enzyme digestion.

	Volume [µl]
Water	Y
DNA sample	X
Buffer	5
Enzyme 1	1
Enzyme 2	1
Total	50

3.1.2. Agarose-gel-electrophoresis

Gel electrophoresis is used to verify the products of restriction enzyme digestion and Polymerase chain reaction (PCR) amplification. The agarose dissolves in Tris base acetic acid EDTA (TAE)-buffer to form the gel. The size of the gel pores depend on the percentage of the agarose added. A 1 % gel forms pores big enough for DNA to migrate and separate samples longer than 400 bp, whereas samples with a shorter length were sparated on a 1.5 % agarose-gel. In order to visualize the fragments migration through the gel and avoid sample dispersion, a loading dye is added to the sample in the ratio 1:6, a DNA ladder is utilized as a reference of fragment sizes. After loading the mixture into the prepared gel, an electric field is applied which leads to separation of the fragments according to their size, structure (supercoiling) and charge. The DNA bands are visualized using an agarose gel imaging system. Bands of the correct size are excised and extracted for further DNA purification. The DNA extraction and purification from agarose gels is performed following the manufacturer instructions of E.Z.N.A® Gel Extration Kit-centrifugation protocol. Basically, the agarose band containing the DNA is dissolved at 60 °C. The DNA is loaded onto a column where it binds to the structure, several washing steps purify the DNA sample until a change in pH-level results in elution of the DNA from the column[23].

3.1.3. Restriction enzyme digestion

Various DNA restriction enzymes recognize different short sequences of base pairs and cut close to them. In general, 2 µg of DNA is incubated with the enzyme and buffer in a total reaction volume of 50 µl. In case of double digestion, a corresponding buffer suitable for both of the selected enzymes is chosen. Water (Y) is used to complete the reaction volumes (table 5). The reaction takes place for three hours at 37 °C. Afterwards, the sample is loaded on a agarose gel and proceeded like described in subsection 3.1.2..

3.1.4. High-Fidelity DNA Assembly

The *NEBuilder HiFi DNA Assembly Cloning Kit* is used to assemble different DNA fragments. The given master mix includes an exonuclease which creates single stranded overhangs at first. These complementary sequences of different DNA fragments anneal, and an included polymerase extends the 3' ends which results in one double stranded DNA fragment.

3.1.5. Ligation

For samples which could not be assembled using the kit outlined above, a ligation can be performed by using the *T4 DNA Ligase* and following its given manual.

3.1.6. Transformation in competent *Escherichia coli* cells and colony PCR

The manufacturer's protocol of *One Shot Competent Cells* is used to transform all products of the *HiFi DNA Assembly* and the ligation into competent *E. coli* cells. A performed heat shock leads to permeabilization of the plasma membrane and allowing the plasmid to enter the bacterial cell. The colony PCR method (See subsection 3.1.1.) is used to determine the successful transformation. The aim of this method is to amplify the transformed plasmid and visualize the product using agarose gel electrophoresis and therefore verify the existence of the plasmid. After picking single colonies from the LB agar plates, a starter culture (50 µl LB medium with selective antibiotic) is incubated for one hour at 37 °C. A volume of 2 µl of starter culture are used for the reaction. To verify of the plasmid carrying the insert, specific primers for the inserted genes are chosen. Furthermore, the *taq* polymerase from (VWR life science) is used as it is cheaper, which requires an elongation time of a minute per 1 kbp DNA amplified and 94 °C to denature the double strands.

3.1.7. Plasmid DNA preparation

After analyzing the results of the colony PCR, the chosen colonies are taken for a liquid LB-medium culture. For this procedure, 50 µl is taken from the starter culture and incubated over night in 7 ml LB-medium. The plasmid DNA is extracted and purified using the E.Z.N.A.® Plasmid DNA Kit Quick Guide and following the man-

ufacturer's instructions. The solutions provided in the kit enable a lysis of cell parts but preserving the plasmid DNA and purifying it[23]. The products are analyzed by sequencing. The samples with the highest quality are chosen for the final preparation. In order to yield enough plasmid for transfection, an overnight culture with 100 ml and 48 µl starter culture from the previous method (see 3.1.6) is incubated at 37 °C. The culture is centrifuged at 4000 rpm for 30 min to spin down the bacteria before perfoming a plasmid maxi-preparation following the manufacturer's instruction of the *NucleoBond® Xtra Midi E*. Concentration of the samples is measured by spectrography.

3.1.8. Sequencing

The plasmid and PCR products quality the construct is sequenced by the department of Entomology, MPI for Chemical Ecology using the method of Sanger sequencing.

3.2. Methods for specific constructs

3.2.1. Constructed plasmid

The desired regions of the two different plasmids pBI-CMV1 and pCMV-TNT were amplified with the usage of the *Phusion taq* polymerase. Primers used for the PCR of pBI-CMV1 were pBI-CMV1_fwd, pBI-CMV1_rev creating a piece of 1378 bp length. The backbone of the designed plasmid was created by amplification of a 2700 bp long DNA region using the pCMVTNT_fwd, pCMVTNT_rev primers. A *HiFi Assembly* then assembled the pieces into one plasmid.

3.2.2. Construct 1: pCMV-BI-hOrco(full length)-OR47a

After linearization of the designed plasmid pCMV-BI by restriction enzyme digestion using the enzymes BamHI-HF and HindIII-HF, a human codon optimized version of the Odorant receptor co-receptor (Orco) was ligated into the plasmid. The specific receptor OR47a was amplified via PCR with the usage of the BI-MTSV5ERSOr47a forward and reverse primers. The construct (pCMV-BI-hOrco) was then cut through restriction enzyme digestion using a EcoRI restriction site. A *HiFi Assembly* was

performed to assemble the plasmid and the receptor OR47a. The colony PCR used to verify the final constuct was performed using the same primers which were taken to amplify the gene of OR47a. In order to check the quality of the product the sequencing was performed after the plasmid was cleaved in two pieces by restriction enzyme digestion using NaeI and XhoI.

3.2.3. Construct 2: pCMV-BI-hOrco(intron)-OR47a

In order to create the co-receptor Orco including the β-globin/IgG intron hOrco was amplified by PCR in two different reactions so that two pieces were created. The primers used were hOrcoExon1_fwd, hOrcoExon1_rev and the corresponding hOrcoExon2 forward and revers. The β-globin/IgG intron was amplified by using chimeric_intron_fwd, chimeric_intron_rev. All three pieces were assembled by performing a *HiFi Assembly*. The specific receptor was amplified like previously described. After linearization of the plasmid (pCMB-BI-hOrco(intron) with EcoRI-HF it was assembled into one with Odorant Receptor (OR)47a. As a verification of the product a colony PCR was performed after the transformation with the usage of the specific OR47 primers. The final construct was digested with NaeI and XhoI before it was sequenced.

3.2.4. Construct 3: pCMV-BI-hOrco(intron)-(intron)OR47a

The receptor OR47a was amplified via *Phusion taq* polymerase reaction using the CI-MTSV5ERSOr47a_fwd and CI-MTSV5ERSOr47a_rev primer couples. The desired β-globin/IgG intron was amplified using the ChimIntr_Or47a_fwd and ChimIntr_Or47a_rev primers and the *Phusion taq* polymerase so it could be assembled in front (5'-3') of the specific receptor OR47a by performing a *HiFi Assembly*. Different primers were taken for this construct to ensure that ends were created suitable to perform a *HiFi assembly* with OR47a and the plasmid. The vector pCMV-BI including hOrco(intron) was linearized by restriction enzyme digestion with the usage of XbaI. Another *HiFi Assembly* was realized to obtain the final product composed of the plasmid and OR47a including the β-globin/IgG intron. The method of restriction enzyme digestion was performed to create two pieces of the final construct by using NaeI and XhoI in order to sequence it.

3.2.5. Construct 4: pCMV-BI-hOrco(full length)-(intron)OR47a

The β-globin/IgG intron and OR47a receptor were amplified as described in the construct above. The co-receptor Orco was ligated in the linearized plasmid pCMV-BI. A *HiFi Assembly* was performed to create the final product consisting of pCMV-BI-hOrco(full length) and OR47a(intron) after the vector was linearized using XbaI. After cleaving the plasmid into two pieces samples could be sequenced.

4. Results

In order to improve the rate of expression in mammalian cells, the *Drosophila melanogaster* genes of interest were codon-humanized. Codons follow a three-bases-code encoding each amino acid. For almost all amino acids there are usually more than one possible nucleotide sequence on the three-bases-code and different organism favor certain codons over anothers. Thus, this is an important fact to consider when expressing genes heterologously outside their original surrounding[24, 25].

To circumvent the problem of trafficking the OR to the outer plasma membrane, in this thesis the OR was tagged with peptides that aim at aiding the trafficking through the intracellular space. First, an endoplasmic reticulum (ER) export motif was added to the OR sequence. This 20 amino acid long sequence is part of the Hyperpolarization-activated cyclic nucleotide-gated 1 (HCN) channel in the retina of humans and it was found to be responsible for ER-forward trafficking[26]. In order to gate the ORX and its co-receptor Orco to the cell membrane after release from the endoplasmic reticulum, a second signal was added for vesical trafficking. The motif used in this case comes from the post-golgi apparatus transport motif of rhodopsin[27, 28]. Rhodopsin plays an important role in vision and is produced in the inner segment of the cell but then appears to be well concentrated on the outer segment[29]. Therefore, I made use of this guiding property in this thesis to facilitate the transport of the receptor to the cell membrane.

The humanized sequences for OR47a and hOrco including the addition of the tags were prepared by Fabio Miazzi.

A bidirectional plasmid pBI-CMV1 was chosen to express both genes at the same time. Due to the fact that it has a origin of replication of low copy number, the promotor of another plasmid (pCMV-TNT) including a high copy number origin of replication was inserted as a backbone. Another reason for the second vector (pCMV-TNT) was an enhancement β-globin/IgG intron, which is known to increase the expression of proteins in mammalian expression systems[30, 31, 32]. Multiple cloning sites were kept to enable fast and easy restriction enzyme digestion and inclusion of other ORs in future studies. Figure 3 represents the two selected vectors

(pBI-CMV1 and pCMV-TNT) and the resulting plasmid pCMV-BI.

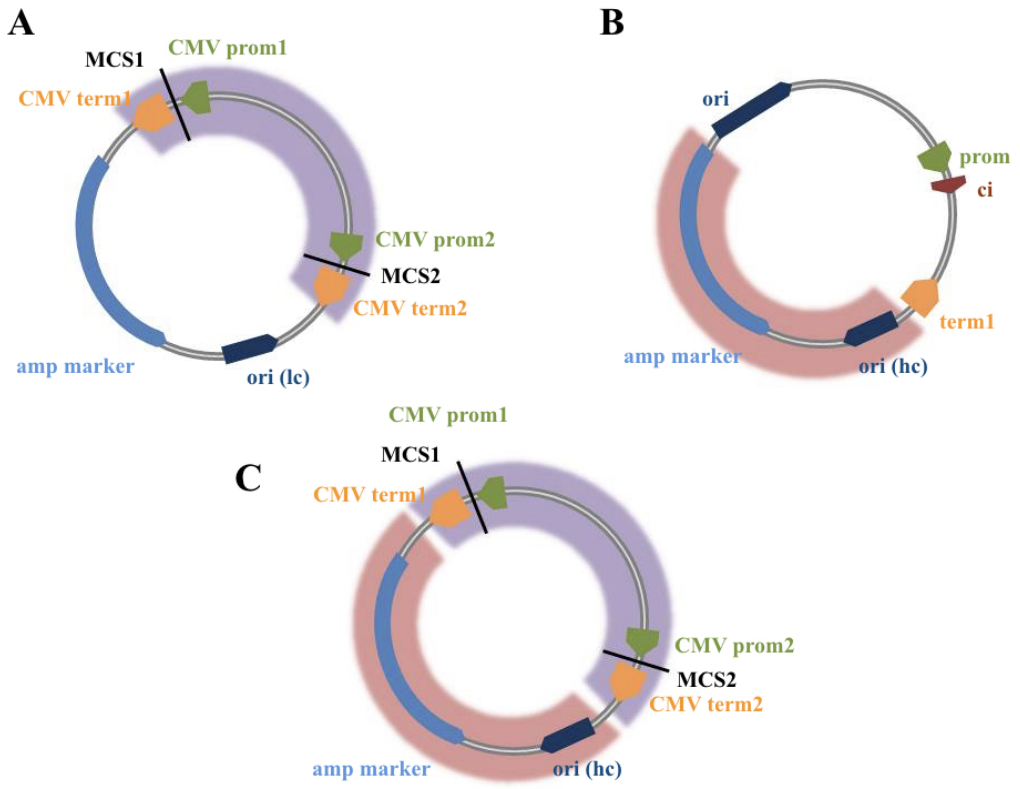

Figure 3.: **A** Representation of the bidirectional plasmid pBI-CMV1. **B** Representation of the plasmid pCMV-TNT. **C** The constructed plasmid pCMV-BI. prom = promotor; term = termination; amp = ampicillin promotor and marker gene; ori = origin of replication (hc = high copy; lc = low copy); ci = chimeric intron; MCS = multiple cloning site.

4.1. pCMV-BI-hOrco(full length)-OR47a

The plasmid pCMV-BI was cut using two restriction enzymes as described in section 3.2.2.. The sample (2) showed a clear and strong band at the correct size of 4000 bp and was therefore used to continue with (see figure 4). After excision and purification of the cut plasmid DNA, a concentration of 30.7 ng/ µl was measured (See appendix A.1 for post-cut agarose-gel picture). The human codon optimized version of the co-receptor Orco was ligated into the plasmid pCMV-BI. Visible figure 5 appendix A are the results of the PCR to amplify the specific receptor OR47a and the restriction enzyme digestion of pCMV-BI-hOrco (full length). Lane 2 shows a strong and clear band for OR47a which appears at the correct length of about 1200 bp. Bands of the digested plasmid including hOrco(full length) in lane 4 appear clear and at the expected size of circa 5500 bp but not as strong as the one of OR47a. Both

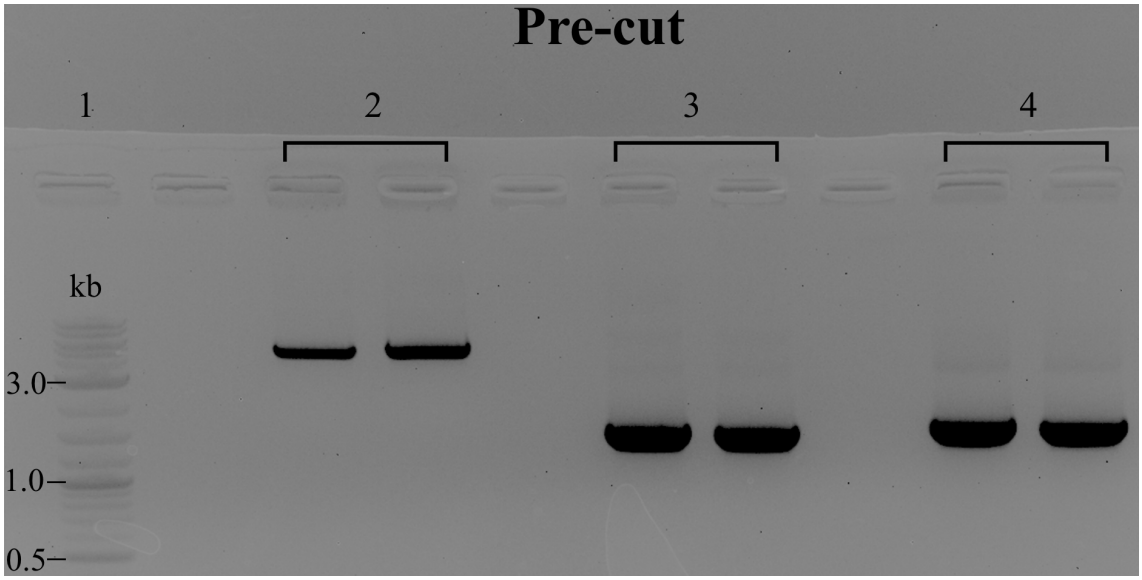

Figure 4.: Successful PCR and restriction enzyme digestion visualized on a agarose-gel.
1 2-Log DNA ladder **2** pCMV-BI **3** hOrco (full length) **4** hOrco (intron).

samples were excised and purified yielding a concentration of 73.2 ng/µl for the specific receptor and 31.0 ng/ µl for the plasmid including the humanized Orco.

As a result of the performed transformation 12 colonies were picked and verified via colony PCR shown in figure 2 in appendix A. Figure 11 appendix A shows the agarose-gel picture where the relevant agarose gel for the first construct is shown in the upper part. Well 1 represents the ladder whereas 2-13 show the different colony PCR results. Due to the fact that all colonies show a band of the amplified specific receptor OR47a the strongest four were chosen to continue with. In the subsequently completed mini-preparation an average concentration of 135 ng/ µl was reached. The digestion of the final construct was successful for all samples showing two bands on the agarose gel after they were cut with two different enzymes (see figure 3 appendix A). Both bands of each samples were excised and purified with an average concentration of 15 ng/ µl.

Through perfoming another PCR, the average concentration of the samples could be increased to 93 ng/ µl. A *advantage taq* polymerase was chosen to ensure correct amplification. The sequence of sample 4 had the best quality and was therefore chosen for maxi-preparation. Measurement of the concentration after the maxi-preparation was performed showed 4686 ng/ µl of double stranded DNA.

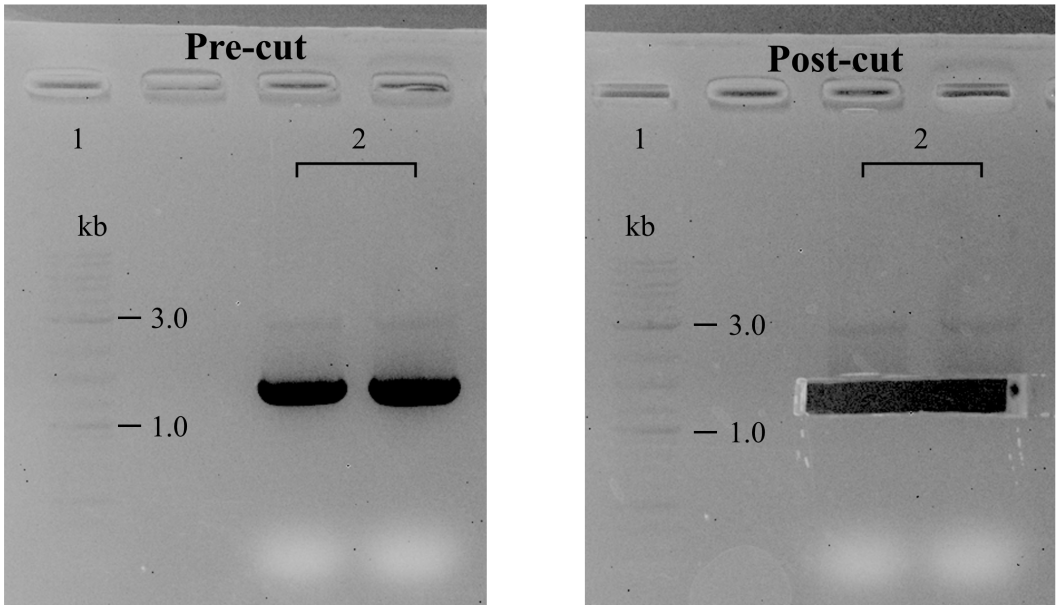

Figure 5.: PCR hOrco-Exon1.
Precut Successfull amplification of the first hOrco part at the approximate size of the expected 1.25 kb **1** 2-log DNA ladder **2** hOrco **Postcut** Excised band of hOrco **1** 2-log DNA ladder **2** Excised band hOrco.

4.2. pCMV-BI-hOrco(intron)-OR47a

In order to create construct 2, the co-receptor was successfully amplified in two pieces. Figure 5 shows the PCR result of the first Orco piece as 2 before and after it was excised (correct expected size of 1248 bp). The purification process yielded a concentration for hOrco-Exon1 of 72.1 ng/ µl. The amplification of hOrco-Exon2 and the β-globin/IgG intron can be seen in the appendix A figure 6. The concentrations reached were 139.9 ng/ µl for hOrco-Exon2 and 108.4 ng µl for the β-globin/IgG intron. Exon 1, 2 and the chimeric intron were then assembled into the plasmid and afterwards digested. Results of the digestion can be seen in an agarose gel picture in figure 5 appendix A (lane 3). The specific receptor OR47a was successfully amplified before to realize construct 1 and the sample therefore reused to create the second construct. After the *HiFi Assembly* and transformation was performed, a colony PCR was used to verify the product. Again, all twelve selected colonies showed a band for the amplified receptor OR47a thus four colonies showing the strongest bands were chosen for the mini-preparation.

Results of the mini-preparation yielded an average concentration of 103 ng/ µl. The performed restriction enzyme digest to cut the plasmid in order to realize the sequencing resulted in an average sample concentration of 13 ng/ µl after both bands were excised and purified. The agarose-gel is visible in figure 6 before bands were excised. The sample in well 3 shows an additional band which was not cut. Due to

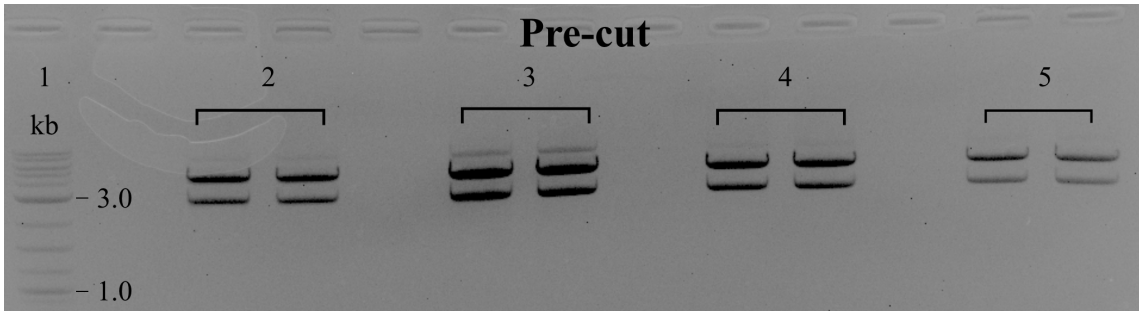

Figure 6.: Agarose-gel picture of a restriction enzyme digestion of construct 2 before bands were excised.
1 2-log DNA ladder **2-5** cut samples.

the fact that a higher concentration is necessary for sequencing a *advantage taq* PCR was performed to increase the yielded product. After this an average concentration of 87 ng/ µl was reached.

The sequencing of both corresponding parts of the plasmids showed that sample 4 is of the best quality. A maxi-preparation of sample 4 (fig. 6) was performed and a final product concentration of 1207 ng/ µl was reached.

4.3. pCMV-BI-hOrco(intron)-(intron)OR47a

The constructed pCMV-BI bearing hOrco(intron) from the previous construct was reused. Therefore only the specific receptor Or47a (see figure 7) and β-globin/IgG intron (data not shown) were amplified with suitable primers to realize a *HiFi assembly* afterwards. The two target regions were purified from an agarose gel yielding a concentration of 77.2 ng/µl for the β-globin/IgG intron and 69.6 ng/µl of Or47a. After the *HiFi assembly* and the transformation was performed 16 colonies were picked the next day. In order to verify the the selected colonies primers amplifying a part of the plasmid were chosen to realize the colony PCR. Expected was a difference in length in case the amplified plasmid part beared the intron and receptor Or47a. Only four colonies were of the correct length (data not shown). Therefore colony 2, 6, 14 and 16 were chosen for mini-preparation which resulted in an average concentration of 161.9 ng/µl Sample 14 and 16 were chosen to continue with and therefore were digested and amplified to enable sequencing (data not shown). Due to the results of the sequences sample 14 was the one which was taken to set up a maxi plasmid preparation. Measurement of the optical density of the final product showed a concentration of 2238.7 ng/µl.

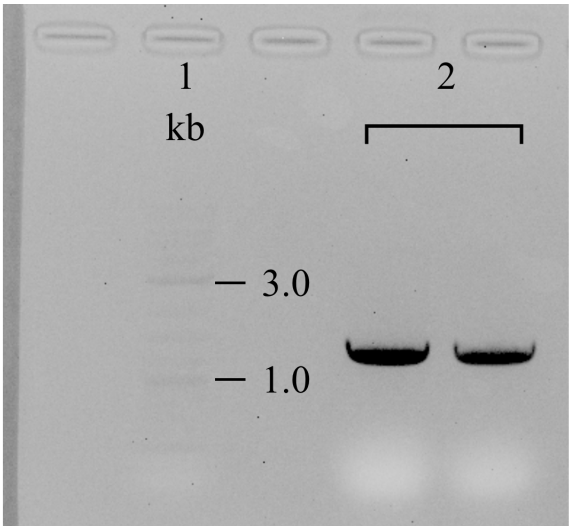

Figure 7.: PCR of OR47a for construct 3.
1 2-log DNA ladder **2** OR47a at expected size of 1.2 kb.

4.4. Calcium imaging results of this project

Based on the advantages and disadvantages of the different techniques, the favored method of odorant receptor expression in HEK293 cells coupled with calcium imaging was chosen. The constructs generating this thesis were tested via Calcium imaging performed by Fabio Miazzi. An adjusted version of the protocol for Ca^{2+} imaging experiments, shown in the appendix B was used.

Calcium imaging experiments which were done up to now, used the plasmid pcDNA3.1(-) and not the tags that were used in this thesis. When co-transfecting OR47a and Orco as respone rate of 2.9 ± 1.05 % to pentyl acetate and 30.23 ± 7.217 % to VUAA1[33]. Adding the different tags which were also used in this thesis already increased the rate of responses to 30.51 ± 6.56 % (pentyl acetate) and 71.76 ± 3.29 % (VUAA1).

Visible in figure 8 are the cells including the different constructs that were tested through calcium imaging using the constructed vector pCMV-BI with OR47 and Orco. The first row labeled A represents OR47a and Orco cloned in pcDNA3.1 without any improvement like it was done and used before. The plasmid (pcDNA3.1(-)) used up to now, is a mammalian expression vector as well but only contains one promotor and multiple cloning site. Therefore a co-transfection was necessary.

Reacting cells are visible in red due to the fluorescence shift of Fura 2 after binding to Ca^{2+}. The only two cells showing an excitement already showed shifted fluorescence before the addition of the odorant. Few cells appear to respond to VUAA1. The graphs shown in figure 9 A gives a similar impression. The black line builds the

mean of the responding cells showing only little response. The peak and therefore reaction to pentyl acetate is almost invisible while the peak to VUAA1 is rather long instead of a sharp peak.

In comparison B figure 8 shows the first plasmid (pCMV-BI-hOrco(full length)-Or47a) constructed for this project. Even though fewer cell appear in the picture over half of them are responding to VUAA1 (B 3). Figure 9 B shows a clear peak after the stimulation with VUAA1 with a Ca^{2+} concentration influx into the cell of over 250 nM.

The construct with the best results is pCMV-BI-hOrco(intron)-Or47a shown in figure 8 C. From the cells shown in the picture only few do not react to stimulation with VUAA1 while also the peak in figure 9 C gives the same impression. The concentration of intracellular Ca^{2+} is almost double (500 nM) of the construct without the intron visualized in B. The response to pentyl acetate also seems to be the highest in this case, compared to other constructs and the vector used before. In comparison to previous experiments, where HEK293 cells were co-transfected with the plasmid pcDNA3.1(-), each carrying either Or47a or Orco (with tags) the response rate was significantly higher. Through co-transfection a response rate of 30.51 $\pm$ 6.56 % to pentyl acetate and 71.76 $\pm$ 3.29 % to VUAA1 has been accomplished[33]. The usage of pCMV-BI as the one transfected vector could increase the rate of response to the same odorants to 76.01 $\pm$ 10.64 % and up to 92.47 $\pm$ 4.03 %.

In order to see if the inclusion of another β-globin/IgG intron in front of the specific receptor Or47a would improve the expression even more construct four was realized, shown in figure 9 D and 8 D. The peak represented by the mean line does not support this presumption. Both peaks and therefore the calcium concentration are smaller than of the ones shown in C. The $[Ca^{2+}]$ seems to be similar to the results of the construct without any β-globin/IgG intron. The calcium imaging pictures shown in figure 8 D underline this result. Here, only few cells are shown to respond to pentyl actetate and even fewer seem to respond to VUAA1. In comparison in figure 8 C a clearly visible difference of response rate is visible between 2 and 3. The number of responding cells in figure 8 D seems to be comparable to the one of A where only very few cells were responding to either stimulant.

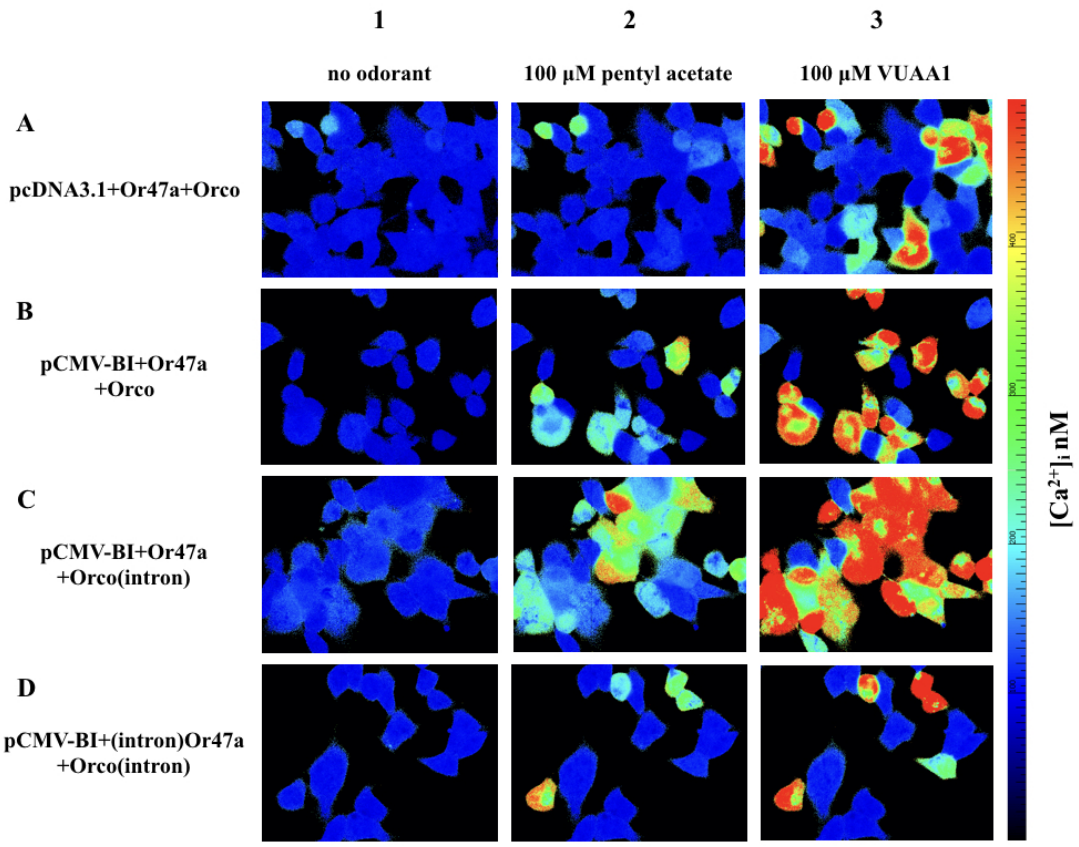

Figure 8.: Calcium imaging of HEK293 cells expressing *Drosophila melanogaster* odorant receptors.
1 cells before adding odorants **2** cells after addition of 100 µM pentyl acetate **3** cells after adding 100 µM VUAA1 **A** Specific odorant receptor Or47a and its coreceptor Orco cloned in pcDNA3.1 **B** Or47a and Orco (full length) cloned in constructed plasmid pCMV-BI **C** Or47a and hOrco including the β-globin/IgG intron in pCMV-BI **D** pCMV-BI with the β-globin/IgG intron in front of Or47a and hOrco (intron)
Cells were stimulated with pentylacetat and VUAA1. (Imaging performed by Fabio Miazzi [33]).

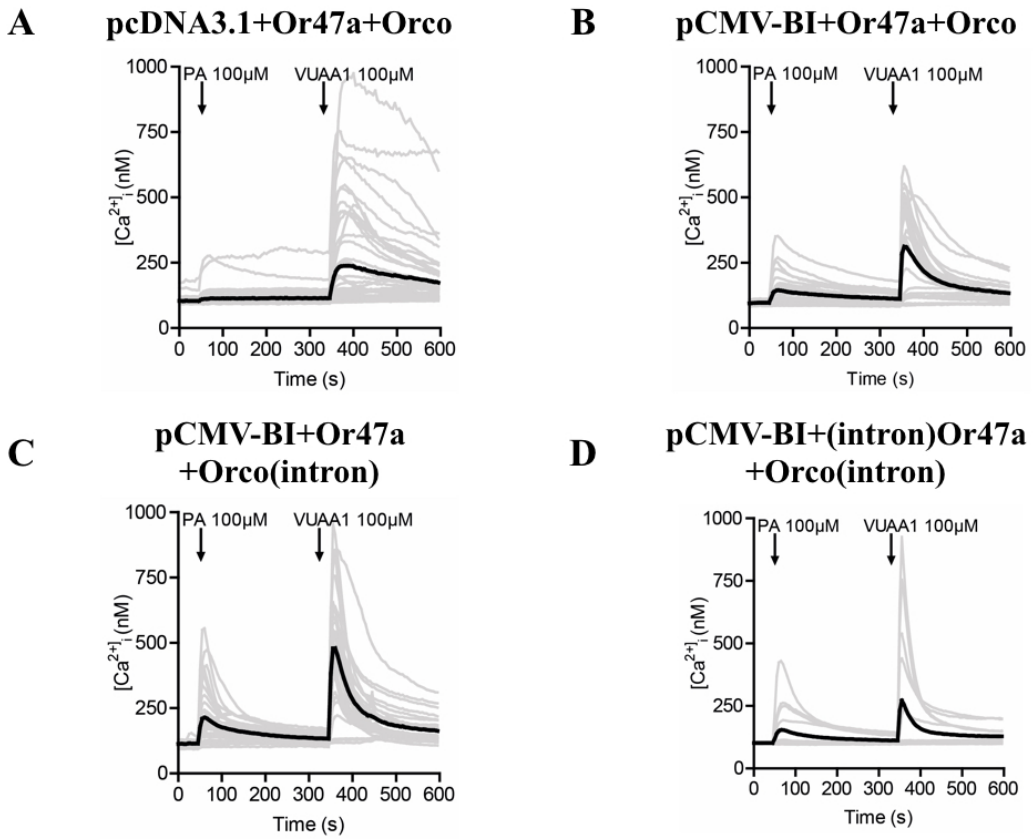

Figure 9.: Graphs showing results of Calcium imaging of HEK293 cells expressing *Drosophila melanogaster* odorant receptors.
A Graph showing the response of cells expressing Or47a and Orco from vector pcDNA3.1 **B** response of cells expressing Or47a and Orco (full length) in pCMV-BI after stimulation **C** cells responding expressing Or47a and hOrco including the β-globin/IgG intron in pCMV-BI **D** stimulated cells expressing Or47a (intron) + hOrco (intron). (Imaging performed by Fabio Miazzi [33]).

5. Discussion

5.1. Techniques used for expression of odorant receptors

Several methods for the research of ORs in insects are well established. Each one has advantages, as well as downsides, and depends on their use and on the aim of the research.

The *in vitro* technique in *Xenopus* oocytes for the functional research on insect receptors is widely spread[1]. One of the major facts contributing to its importance is the high rate of expression after injecting RNA or complementary DNA (cDNA), and the correct post-translational modification of proteins[34, 35]. However, frog eggs were not suitable for high-throughput screening, as electrophysiology has to be manually performed on each egg, and the responses to ligands show high variablity until automated system were developed[36]. Automated systems like the *OpusXpress*, which allows the screening of up to eight oocytes eggs at the same time, and which is shown to have higher accuracy than 96 well plates[34]. The *Roboocyte* is a fully automated platform, performing all steps from injection to the electrophysiological recording[35]. Even though *Xenopus* oocytes fulfills criteria for a good research method, it does not represent the natural environment of *Drosophila melanogaster* odorant receptors, which may be the cause of the varying results; particularly because their eggs are non-neuronal cells[1].

The most natural and accurate surrounding to study *Drosophila* receptors is, in fact the vinegar fly itself[1, 37]. In the "empty neuron" system described before, ORs are expressed in their native environment and tested by single sensillum recording[1, 12]. This represents the best *in vivo* technique to study ORs bringing the benefits of the natural neuronal cell environment. However, the method of single sensillum recording is not suitable for high-throughput screening, and has other disadvantages like the demand of creating specific transgenic flies, to ectopically express each given OR, making it a time consuming technique[36, 1]. Another be-

fore mentioned *in vitro* method with a rather short set-up time is the expression of *Drosophila* odorant receports in insect or mammalian cells[1]. The expression in e.g. ovary cells of *Spodoptera frugiperda* does not require co-expression with Orco since an endogenous Orco orthologous gene expressed, is existing in the cell[38, 39]. In contrast to *Xenopus* oocytes, *Spodoptera frugiperda (Sf9)* offers a micro-environment closer to the native surrounding of *Drosophila*, while still being non-neuronal cells. Due to their insect origin, J.A. Corcoran et al. stated in their 2014 report, that *S. frugiperda* may have odorant binding proteins allowing the correct trafficking of odorants and their interaction with the OR in the cell membrane. Furthermore odorant binding proteins (OBPs) are thought to improve the sensitivity and specifity of ORs to odorants[1]. One of the biggest disadvantages of *Sf9* cells is, that the only method to study functional insect ORs is electrophysiology. In contrast HEK293 cells enable not only electrophysiological recording but also Ca^{2+} imaging.

HEK293 is a very well known mammalian cell line chosen for the purpose of heterologous expression[36, 1]. Even though it is neither a neuronal cell line nor the natural surrounding, like *Drosophila* ORs, it has a bigger range of application techniques. Furthermore cyclic adenosine monophosphate (cAMP) can be used for imaging purposes in HEK293 cells. With its potential for high-throughput screening in 96-well plates, the ability to express recombinant proteins and the little inter-/intracellular assay-variation, HEK293 cells is the best suitable cell line for research[36]. J.A. Corcoran et al. 2014 introduced a method to express odorant receptors of the pest moth *Epiphyas postvittana*, creating a stable cell line of HEK293 cells. The vectors chosen were pcDNA^{TM}4/TO and pcDNA^{TM}5/TO which allow expressing genes in eucaryotic cells in a inducible manner[40, 41]. The inducible expression is one of the advantages in this case since the expression of Orco seems to have negative effects on the health of HEK293 cells. Therefore a possible repression of Orco might be useful. Several other plasmids have been constructed and tested. One is the pcDNA^{TM}5/FRT, using the Flp-IN system, which allows an integration of the gene of interest in the host cells genome and therefore an improvement of expression[42, 43]. All of the mentioned vectors use a specific promotor called CMV (human cytomegalovirus) suitable expression in mammalian cells. Secondary non-targeted integration supposedly increases the gene expression[36]. The results J.A. Corcoran et al. (of 2014) indeed show varying Orco expression and variable response to its agonist VUAA1. Notably, the different cells used to express show varying results regarding the ”tuning” of the receptors. While one OR seemed to be widly tuned in Sf9 cells, the same receptor seemed more narrowly tuned in HEK293 cells[36]. A possible reason for this phenomenon could be the difference of their co-receptors. As mentioned before Sf9 cells make use of their endegenous Orco-like

co-receptor which does not have 100 % sequence similarity to the one of other insects like *Drosophila melanogaster*[36, 1]. In contrast to the described advantages the time needed to establish a stable HEK293 cell line, regularly about three months[36], is a counter argument.

Based on the described up- and downsides of the systems used to study insect ORs, introducing a method to transiently express ORs in mammalian HEK293 cells, using one plasmid and test odors via Ca^{2+} imaging was the aim of this thesis. All in all the constructed plasmid is a great improvement for the research method on ORs. Multiple problems could be solved in this project. While the tags added to the receptor genes enhanced the correct trafficking of the receptor to the cellular membrane, the β-globin/IgG intron to increased the expression rate. A co-transfection of two plasmids is not required anymore as only one vector is used to express both hOrco and OR, which increased the rate of transfected plasmid into HEK293 cells. A major issue of co-transfection of two plasmids is the possible uneven ratio of expression, of the two genes of interest. By only introducing one plasmid to the host cell both genes, the ORX and Orco should be expressed at the same level and therefore give a more consistent response. In conclusion of the represented by the results of testing via Ca^{2+} the constructed vector itself and the OR combined with the different tags already improve the expression. The β-globin/IgG intron mentioned before seems to improve the expression as expected. The responses reached in this thesis are comparable to those of a stable cell line without the time consuming disadvantage of generating a stable cell line. Every construct tested in this project seemed to have higher expression than the before used pcDNA3.1 plasmid. However the plasmid pCMV-BI carrying hOrco including the β-globin/IgG intron and the specific receptor OR47a resulted in the highest concentration of intracellular calcium ions, making it the best construct in this thesis.

5.2. Outlook

The vector constructed for this project enhanced the expression of insect OR47a/hOrco in HEK293 cells compared to previous studies. The high number of cells responding enable automated calcium imaging in 96-well plates, which opens the door to high-throughput screens of new ligands and deorphanization of ORs.

Furthermore, to generalize the findings of this thesis other receptors, e.g. the broadly used OR22a could be subcloned in pCMV-BI-hOrco(intron). Since the Hansson department of evolutionary neuroethology works with other insects, pCMV-

BI could be useful for studies on ORs of e.g. the hawk moth *Manduca Sexta*. The constructed plasmid is a versatile tool to perform one step cloning of insect odorant receptors in mammalian cells. By now, a second receptor (OR56a) has been shown to function using the constructed plasmid of this thesis in HEK293 cells, and with this thesis, built the basis for a manuscript which I am co-author of[33].

6. Abstract

The heterologous system of expression is one of the canonical methods to study structure-function of proteins. However the expression of insect odorant receptors (ORs) has proven to be problematic, moreover the co-transfection of cells with, respectively of two plasmids, carrying the genes for the ligand specific OR and its co-receptor Orco, results in low expression rates.

The aim of this thesis was to design an optimized vector to improve the expression of ORs in mammalian HEK293 cells. The sequences of genes of interest were humanized and cloned in mammalian expression vectors. A bidirectional plasmid was used, in order to co-express two genes of interest in one plasmid, which increases the rate of expressing cells. Additionally a β-globin/IgG intron was incorporated to improve the expression rate even further. To evaluate this new tool, the transfection rate and the functional expression of a *Drosophila* OR complex were compared to two plasmid co-transfection. I found that transfected HEK293 cells expressed the ORX/Orco complex of interest and responded to odors when tested in Ca^{2+} imaging experiments. The results propose the designed vector pCMV-BI as an optimal tool for heterologous expression of insect ORs. The construct developed in this report can be employed in automated calcium experiments, as well as in high-throughput screening.

7. Zusammenfassung

Die heterologe Expression ist eine der anerkannten Methoden, um die Funktion von Proteinen zu erforschen. Allerdings hat sich die Expression von odorant Rezeptoren (OR) als problematisch erwiesen, da durch eine Expression zweier Gene (liganden spezifischer OR und Korezeptor Orco), mittels einer Kotransfektion mit zwei Plasmiden nur geringe Expressionsraten erreicht werden.

Ziel dieser Bachelorarbeit war es, einen optimierten Vektor zur Verbesserung der Expression, von ORs von Insekten in HEK293 (human embryonic kidney) Zellen zu konstruieren. Die Sequenzen der erwünschten Gene wurden humanisiert und in einen Säugetier Expressionsvektor kloniert. Eine Steigerung der Expressionsrate wurde durch die Nutzung eines bidirektionalen Plasmiden, welcher die zwei Gene exprimiert erreicht. Um die Expression weiter zu steigern, wurde ein β-globin/IgG Intron eingearbeitet. Um dieses neue Tool zu evaluieren, wurde die Transfektionsrate und die funktionelle Expression mit der herkömmlichen Kotransfektion mit zwei Plasmiden verglichen. Ich fand heraus, dass transfizierte HEK293 Zellen den ORX/Orco Komplex exprimierten und in Calcium Imaging Experimenten auf die getesteten Gerüche reagierten. Die Expressionsrate, welche für diese Bachelorarbeit erreicht wurde, macht den konstruierten Vektor pCMV-BI zu einem optimalen Werkzeug, für die heterologe Expression von Insekten ORs. Das hier entwickelte Konstrukt kann sowohl in automatisierten Calcium Imaging Experimenten, als auch in high-throughput Screenings verwendet werden.

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A. Supplementary tables and figures

Tables

Table 6.: Concentrations of purified samples after restriction enzyme digestion.

Construct	Concentration [ng\µl]
pCMV-BI	32.2
hOrco (full length)	87.0
hOrco (intron)	87.6

Figures

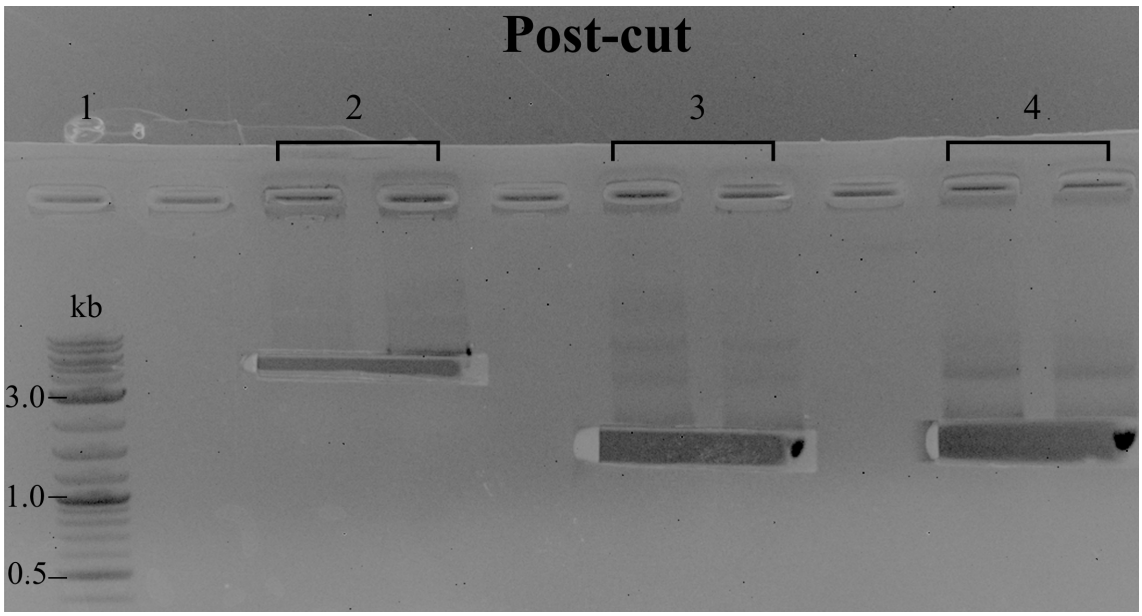

Figure 10.: Post-cut agarose-gel picture of a successful restriction enzyme digestion.
1 2-Log DNA ladder **2** pCMV-BI **3** hOrco (full length) **4** hOrco (intron) at expected size of 133 bp.

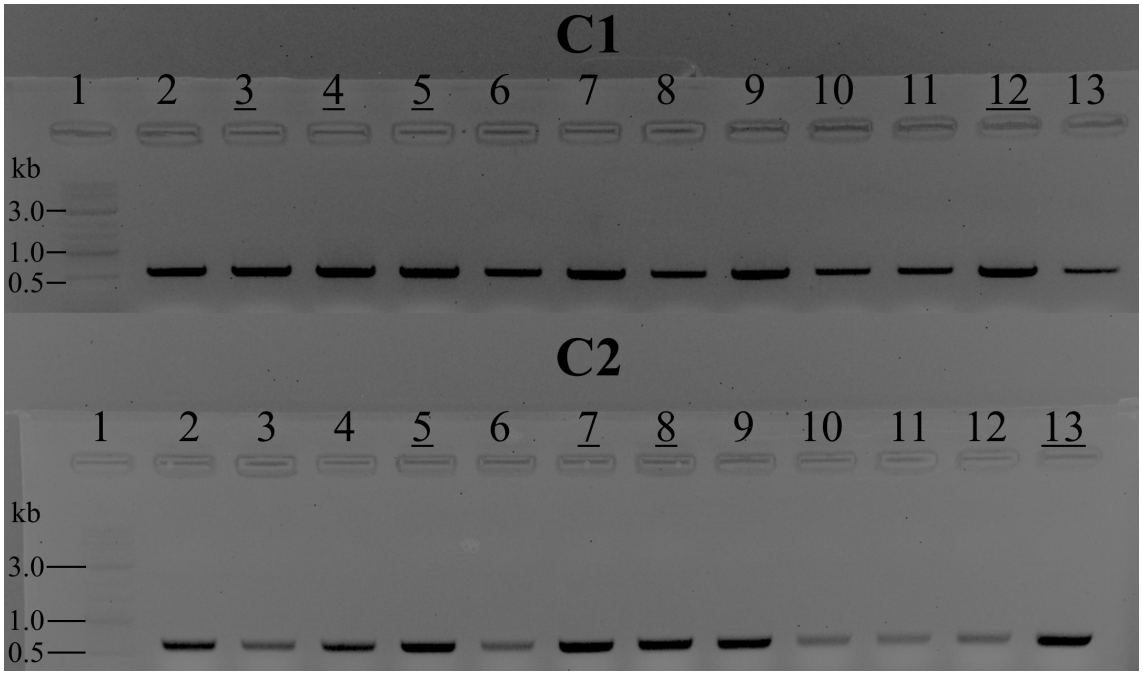

Figure 11.: Agarose-gel picture of a colony PCR.
C1 Construct 1 (pCMV-BI-hOrco(full length)-OR47a) **C2** Construct 2 (pCMV-BI-hOrco(intron)-OR47a).

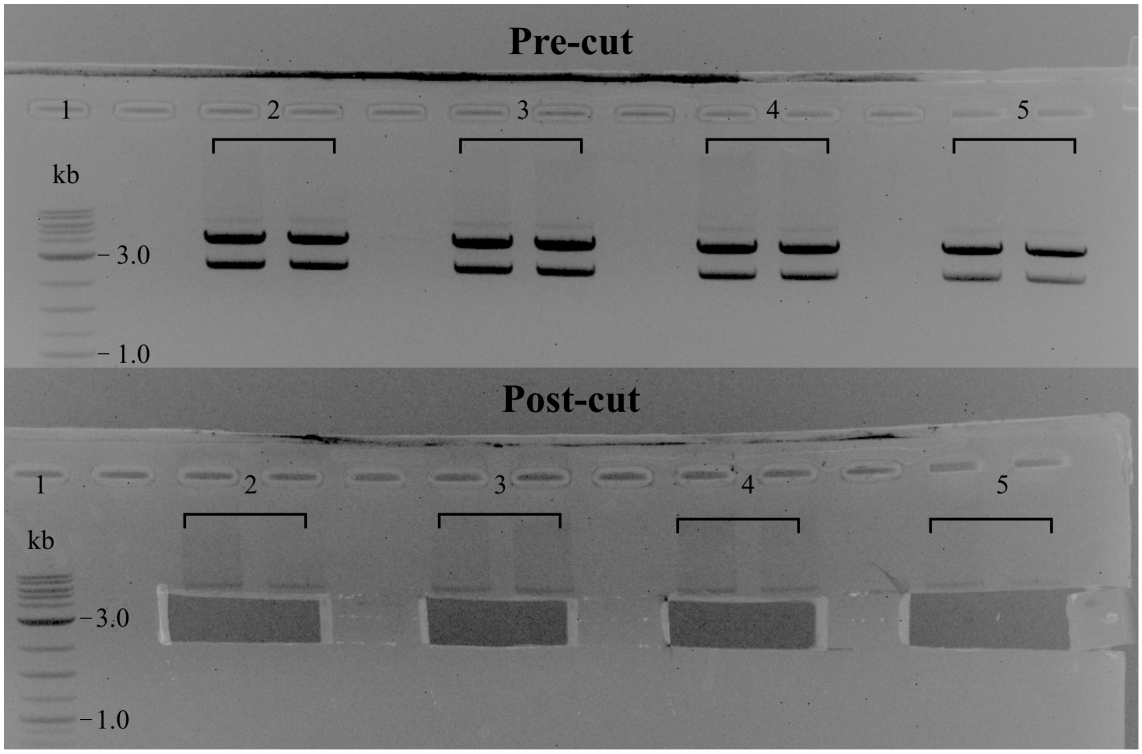

Figure 12.: Agarose-gel picture of a restriction enzyme digestion of pCMV-BI-hOrco(full length)-OR47a.
1 2-log DNA ladder **2-5** cut samples.

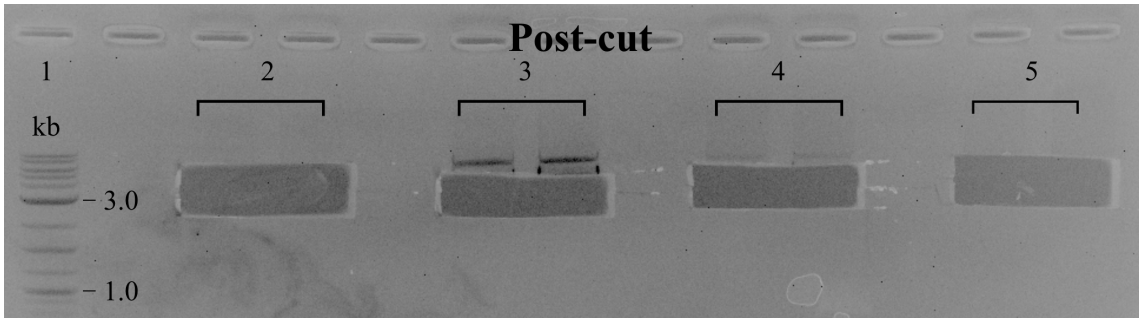

Figure 13.: Agarose-gel picture of a restriction enzyme digestion of pCMV-BI-hOrco(intron)-OR47a after bands were excised.
1 2-log DNA ladder **2-5** cut samples.

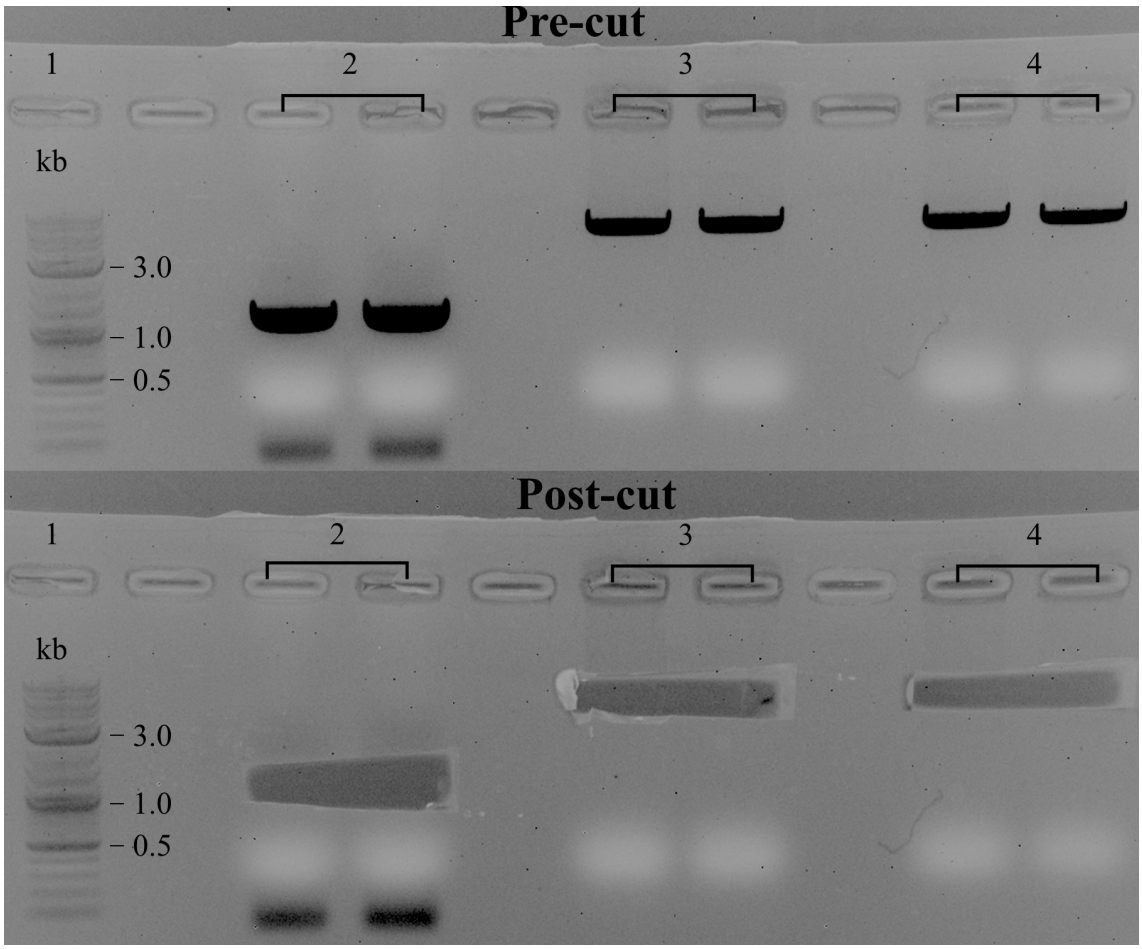

Figure 14.: Agarose-gel picture PCR amplification and restriction enzyme digest.
1 2-log DNA ladder **2** amplified specific receptor OR47a **3** successfully cut vector pCMV-BI-hOrco(intron) **4** successfully cut plasmid pCMV-BI-hOrco(full length).

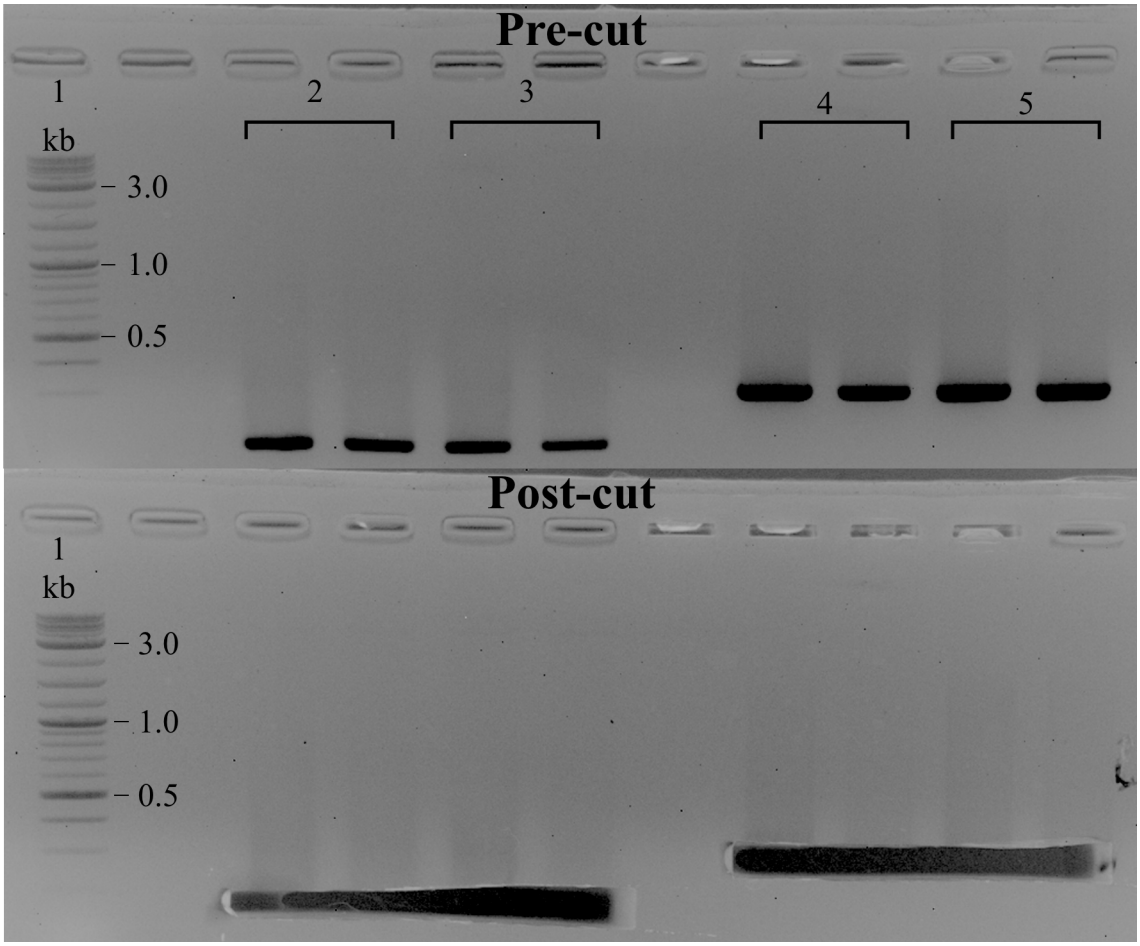

Figure 15.: Agarose-gel picture PCR amplification.
1 2-log DNA ladder $\mathbf{2+3}$ β-globin/IgG intron $\mathbf{4+5}$ hOrco-Exon2.

B. Protocol: Calcium Imaging of HEK293 transfected cells

ICE-16 - Heterologous Expression Practical Course

Protocol for Calcium Imaging of HEK293 transfected cells

Before starting

Cell culture and transfection procedures

- Cultivate HEK293 cells in DMEM/F12 1:1 medium plus 10% fetal calf serum (FCS) on cell culture flasks at 37°C and 5% CO_2;
- Electroporate 5×10^5 cells/well with 0.3 µg/well DmelOrco in pcDNA3.1(-) plasmid;
- Plate the cells on 0.01% poly-L-lysine coated coverslips (5×10^5 cells/well) in a 24 wells plate;
- Cells can be imaged 24 hours post electroporation;

Preparation of stocks and solutions

- SES (Standard Extracellular Solution): 140mM NaCl; 5mM KCl; 1mM $MgCl_2{\cdot}6H_2O$; 1mM $CaCl_2$; 10mM HEPES; 10mM Glucose, in double distilled water. The solution is equilibrated to pH = 7.4 using 1N NaOH and 0.1N HCl solutions in double distilled water. Nominal osmolarity 297 mOsm/l; for long term storage freeze at -20°C, for short term usage, keep at +4°C.
- Fura2-AM stock, 1mM: each vial of 50 µg is dissolved in 50 µL of DMSO. It is then divided in 5 µL aliquots in individual 1.5 mL tubes and stored at -20°C.
- VUAA1 stock: 100 mM in DMSO, stored at -20°C.

Getting started

- Bring required solutions (Opti-MEM, SES) to room temperature and adjust the pH of SES to 7.4;
- Incubate each dish plated with cells in the dark in a CELLSTAR 35mm cell culture dish with 5 µL of 1mM Fura-2 dissolved in 1mL Opti-MEM (5µM final concentration) for 30 minutes at room temperature; ❗ Perform this step in the dark to avoid photo bleaching of the Fura2-AM sensor!
- Prepare the VUAA1 working solutions dissolving the necessary amount of the 100 mM stocks in room temperature SES to reach the concentration required (here we will use 100 µM). ❗ Prepare fresh, do not store working solutions!
- When the incubation time is over, discard the Fura-2 containing Opti-MEM and gently wash the cells 3 times with 1 mL of SES aspirating and introducing the liquid close to the border of the plate, away from the cells;
- After washing keep the cells in SES (3mL);

- Turn on the setup (monochromator Polychrome V, imaging control unit, camera, PC) and prepare the imaging protocol file to load on TillVision software (Version 4.5, Till Photonics);

Ca^{2+}-imaging protocol using Fura2-AM

- Focus the cells under transmission light with the 40x water objective and select a suitable patch of cells. ❗ Perform these steps in the dark, avoid using a high light intensity and limit the exposure time to avoid photo bleaching!
- Once the cells are on focus take a snapshot of the cell patch under transmission light (50 ms exposition time), 340 and 380 nm light (150 ms exp. time);
- Start the imaging protocol [60 cycles, 5 sec for cycle, excitation at 340 and 380 nm, 150 ms excitation time each];
- Apply 100 µl of the VUAA1 working solution directly on the rim of the objective using a pipette;
- Screen the imaging setup from light until the end of the imaging protocol;
- Select a background area (without cells) in each of the 340 and 380 imaging videos. ❗ The background area in the two videos must be selected in the same area of the plate to avoid artifacts in the calculation of the $[Ca^{2+}]_i$;
- Calculate the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using the Ratio tool of TillVision software; ❗ This step requires a pre-calibration of the setup!
- Use the Selection tool of TillVison to select regions of interests (ROIs) in correspondence of the responding cells;
- Export the selected data for analysis using the I/O menu.

Setup

Monochromator (Polychrome V, T.I.L.L. Photonics, Gräfelfing, Germany)

Imaging Control Unit (T.I.L.L. Photonics, Gräfelfing, Germany)

Sensicam CCD camera (PCO, Kelheim, Germany)

Axioskop FS microscope (Carl Zeiss, Jena, Germany)

Water immersion objective (LUMPFL 40xW/IR/0.8; Olympus, Hamburg, Germany)

Emitted light is separated by a 400 nm dichroic mirror and filtered with a 420 nm long-pass filter.

Free intracellular Ca^{2+} concentration calculation

$$[Ca^{2+}]_i = K_{eff} \frac{\mathrm{R} - R_{min}}{R_{max} - R}$$

R = ratio between image pairs obtained by excitation for 150 ms at 340 nm and 380 nm (after background correction!!!).

K_{eff}, R_{min}, and R_{max} = constants determined with a calibration procedure using ionomycin permeabilized cells.

In our case the values of K_{eff}, R_{min}, and R_{max} are 1.95 μM, 0.2, and 5.3, respectively.

9. Erklärung

Ich versichere, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die inhaltlich oder wörtlich aus Veröffentlichungen stammen, sind kenntlich gemacht. Diese Arbeit lag in der gleichen oder ähnlichen Weise noch keiner Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht.

Hiermit erkläre ich mich mit der Einsichtnahme in meine Abschlussarbeit im Archiv der Bibliothek der EAH Jena ~~einverstanden~~ / nicht einverstanden.

Ort, Datum Unterschrift

10. Sperrvermerk

Die vorgelegte Bachelorarbeit/Masterarbeit mit dem Titel „An optimized vector for the functional expression of insect odorant receptors“ beinhaltet vertrauliche Daten und Informationen des Unternehmens Max Planck Institute for Chemical Ecology.

Diese Bachelorarbeit/~~Masterarbeit~~ darf nur vom Gutachtern sowie berechtigten Mitgliedern des Prüfungsausschusses bzw. der Prüfungskommission eingesehen werden. Eine Vervielfältigung und Veröffentlichung der Bachelorarbeit/~~Masterarbeit~~ ist auch auszugsweise nicht erlaubt.

Dritten darf diese Arbeit nur mit der ausdrücklichen schriftlichen Genehmigung der/~~des~~ VerfasserIn und des Unternehmens zugänglich gemacht werden.

Ort, Datum Unterschrift

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