Complex Encoding of Olfactory Information
by Primary Sensory Neurons

Lu Xu

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY
2020
ABSTRACT

Complex Encoding of Olfactory Information by Primary Sensory Neurons

Lu Xu

The encoding of olfactory information starts from the interaction between odorant molecules and olfactory sensory neurons (OSNs). In mouse, one mature olfactory sensory neuron (OSN) almost exclusively expresses one out of ~1,000 odorant receptors (ORs). The relationship between odorants and ORs is promiscuous: one odorant can activate multiple ORs and one OR can be activated by many odorants.

This combinatorial olfactory coding scheme is fundamental, but not sufficient to fully understand the peripheral encoding of odor mixtures. Almost all naturally-occurring smells consist of many different odorous compounds; for example, rose has more than 275 volatiles and roasted coffee has 850. It is well appreciated in psychology and perfumery that different components in an odor blend can affect each other, producing modulation effects. However, these effects are often considered to be the results of higher center processing, while odor interactions at the peripheral level have not been comprehensively measured.

To evaluate peripheral neuronal responses to odor blends, it is necessary to profile the response patterns of a large population of OSNs while the responses of each individual OSN can be resolved. Conventionally, this has been achieved by imaging OSNs acutely dissociated from the olfactory epithelium with a regular epi-fluorescent microscope. In Chapter 2 of this thesis, such method was utilized to characterize the response patterns of three groups of bio-isosteres. This study reveals that OSNs discriminate odors primarily based on their topological properties rather than chemical properties.
Chapter 3 investigates the modulation effects of Hedione, a chemical that has been widely used in perfumery for 60 years. Hedione is psychophysically known as an enhancer that brings up the volume of floral and citrus odors, but the underlying mechanism remains largely unknown. Our study showed that Hedione could both enhance and inhibit odor responses in peripheral neurons, with inhibition being the dominant effect. Moreover, dose-dependent analyses have shown that odorant receptors with lower binding affinity are more prone to inhibition, leading to the hypothesis that Hedione may act as a weak antagonist, which highlights the scent of the leading compound through contrast enhancement.

However, the usefulness of the cell imaging method utilized in Chapter 2 and 3 was limited by its low throughput (300 cells per field of view) and cell damage during digestion. Taking advantage of a new advance in microscopy, Swept Confocally Aligned Planar Excitation (SCAPE), I was able to perform 3D volumetric imaging on the intact olfactory epithelium of OMP-CRE+/− GCaMP6f−/− mice with a perfused half-head preparation. This method is capable of recording over 10,000 OSNs simultaneously with high spatial and temporal resolution. The process of establishing the imaging protocol and data analysis pipeline has been detailed in Chapter 4.

Chapter 5 characterizes OSN responses to odor blends using the SCAPE microscopy. A large number of responding cells showed inhibited or enhanced responses to odor mixtures compared with responses to each individual component. Eight structurally and perceptually distinct chemicals were tested, all shown to act as antagonists or enhancers to some extent. Compared with a monotonically additive coding scheme, the presence of widespread modulation effects could diversify the output, thereby increasing the capacity of the olfactory system to distinguish complex odor mixtures.
Taken together, these results show that olfactory information is subject to widespread modulation in the olfactory epithelium. This unusual complexity at the primary receptor level implies an information coding strategy different from those utilized by visual and acoustic systems, where complex interactions among stimuli only occur at higher levels of processing. Further experiments are needed to explain the mechanisms at the molecular level and to link peripheral neuronal responses to psychophysics and behavior.
# Table of Contents

List of Figures ................................................................................................................................. v

List of Tables ..................................................................................................................................... ix

Acknowledgements ........................................................................................................................... x

Chapter 1  Introduction ..................................................................................................................... 1

1.1. Anatomical and molecular basis of the peripheral olfactory system .................................... 1

1.1.1. Functional anatomy of the olfactory epithelium ................................................................. 1

1.1.2. Odorant receptors and the one-neuron-one-receptor rule .................................................. 3

1.1.3. OR classification and zonal expression .............................................................................. 4

1.1.4. OSN signaling transduction ............................................................................................... 6

1.2. Encoding of olfactory information .......................................................................................... 8

1.2.1. Navigating the odor space ................................................................................................. 9

1.2.2. The molecular receptive range of ORs ............................................................................ 12

1.2.3. The complexity of olfactory coding ................................................................................. 13

Chapter 2  The Medicinal Chemistry Principles behind Peripheral Encoding of Floral Odors 15

2.1. Introduction to medicinal chemistry ......................................................................................... 15

2.2. Experiment Design .................................................................................................................. 16

2.3. Methods .................................................................................................................................... 17

2.3.1. Chemicals .......................................................................................................................... 17
Chapter 2 Exploring the Modulation Effects of Hedione and Hedione-Like Modulators

2.3.2. Animals and tissue collection .......................................................... 18

2.3.3. Calcium imaging....................................................................................... 19

2.3.4. Data analysis ............................................................................................ 19

2.4. Results ........................................................................................................... 20

2.5. Discussion ....................................................................................................... 22

Chapter 3 Exploring the Modulation Effects of Hedione and Hedione-Like Modulators

3.1. Introduction to the Hedione effect ............................................................... 26

3.2. Methods .......................................................................................................... 27

3.2.1. Animals and tissue collection ................................................................. 27

3.2.2. Calcium imaging.......................................................................................... 27

3.2.3. Data analysis .............................................................................................. 28

3.3. Results ............................................................................................................. 29

3.3.1. Probing the modulation spectrum of Hedione ........................................ 29

3.3.2. Dose-response analyses of the Hedione effect........................................ 31

3.3.3. Characterizing molecular features critical for the Hedione modulation effects ..... 36

3.4. Discussion ....................................................................................................... 46

Chapter 4 Development of SCAPE Microscopy-based OSN Imaging Technique

4.1. Introduction to SCAPE microscopy ................................................................. 48

4.2. Experimental design and Methods ............................................................... 50
5.3.2. Dose response analysis of inhibition ................................................................. 76
5.3.3. Dose response analysis of enhancement ............................................................ 78
5.3.4. Responses profile of odor set 2 ................................................................. 81
5.3.5. Additional modulators of ACE responses ........................................................ 87
5.4. Discussion ........................................................................................................... 91

Chapter 6 Discussion and Future Work........................................................................ 97

6.1. Continuation of the Hedione project ................................................................. 98
6.2. Characterizing odor responses at single receptor level ......................................... 98
6.3. Optimizing the data processing pipeline ............................................................. 99

References ............................................................................................................. 100

Appendix A: Supplementary Materials .................................................................. 109

A.1 CAD for mouse hemi-head perfusion chamber .................................................... 109
A.2 SCAPE imaging of mouse olfactory epithelium activities .................................... 110

Appendix B: Publications and Presentations Related to this Thesis ..................... 111

B.1 Peer reviewed publications ............................................................................... 111
B.2 Conference presentations .................................................................................. 111
List of Figures

Figure 1. Structure of the olfactory epithelium................................................................. 2

Figure 2. Zonal expression of odor receptors in mouse....................................................... 5

Figure 3. Signal transduction in mammalian OSNs............................................................ 6

Figure 4. Henning’s smell prism......................................................................................... 10

Figure 5. Zarzo’s odor cube showing common perfumery descriptors............................. 11

Figure 6. Time course of an individual OSN.................................................................... 20

Figure 7. Floral panel data summary.................................................................................. 21

Figure 8. Hierarchical clustering analysis result of the heterocyclic compound panel........ 23

Figure 9. Hierarchical clustering analysis result of the ester panel................................. 24

Figure 10. Time courses of two individual OSNs showing the modulation effects of Hedione. 29

Figure 11. Diversified modulation effects of Hedione on floral odors................................. 30

Figure 12. Dose-dependent cell recruitment by citral......................................................... 31

Figure 13. Dose-dependent modulation effects of Hedione............................................... 32

Figure 14. Time course of an individual OSN showing dose-dependent inhibition by Hedione. 32

Figure 15. Hedione effects on OSNs with various citral affinities..................................... 34

Figure 16. Hedione effects on OSNs with various eugenol affinities................................. 35

Figure 17. Structures of Hedione stereoisomers ................................................................ 36
Figure 18. Time course of an individual OSN showing the modulation effects of Hedione stereoisomers................................................................. 37

Figure 19. The agonistic and modulation effects of Hedione and its stereoisomers. ............ 38

Figure 20. The agonistic and modulatory effects of Hedione and dehydro-hedione............. 40

Figure 21. Structures of Hedione and its fragmental analogues. ..................................... 41

Figure 22. Time courses of two OSNs showing the effects of Hedione and its fragmental analogues. ........................................................................................................................................... 41

Figure 23. The agonistic and modulatory effects of Hedione and its fragmental analogues........... 42

Figure 24. Chemical structures of Hedione and Fructalate.................................................. 43

Figure 25. Time courses of individual OSNs showing the effects of Hedione and Fructalate..... 44

Figure 26. The agonistic and modulatory effects of Hedione and Fructalate....................... 45

Figure 27. Schematic of the SCAPE system........................................................................ 50

Figure 28. Schematic of intact olfactory epithelium imaging platform for SCAPE microscopy. 52

Figure 29. Custom-designed user interface for supervised piece-wise registration. ............. 55

Figure 30. Demonstration of two non-rigid registration methods........................................... 56

Figure 31. Schematic of preparing inputs for the 2D convolutional neural network.............. 58

Figure 32. Custom-designed GUI for component validation................................................. 59

Figure 33. High-resolution scanning of the intact olfactory epithelium using SCAPE microscopy. ...................................................................................................................... 60

Figure 34. Odor-evoked GCaMP6f activities recorded using SCAPE microscopy. ............... 61
Figure 53. Dose-dependent suppression of dorisyl by 100μM isoraldeine........................................ 85

Figure 54. The effect of 100μM isoraldeine as an antagonist....................................................... 85

Figure 55. Dose-dependent enhancement of dorisyl by 100μM isoraldeine. ............................... 86

Figure 56. The effect of 100μM isoraldeine as an antagonist....................................................... 86

Figure 57. Chemical structures of acetophenone and odors tested as modulators......................... 87

Figure 58. Heatmap of normalized peak responses to acetophenone and four modulators......... 88

Figure 59. Normalized response heatmap of suppressed OSNs. .................................................. 89

Figure 60. Time courses of four OSNs showing suppression....................................................... 89

Figure 61. Normalized response heatmap of enhanced OSNs..................................................... 90

Figure 62. Time courses of four OSNs showing enhancement................................................... 91

Figure 63. Diversified coding capacity through modulation........................................................ 93

Figure 64. CAD drawing of mouse hemi-head perfusion chamber............................................. 109
List of Tables

Table 1. Floral panel compounds ................................................................. 17
Table 2. Solutions for acute OSN culture preparation ................................ 18
Table 3. Top 20 e-Dragon descriptors of odor discrimination in the ester panel. 25
Table 4. Comparison of Hedione isomers as agonists ............................... 39
Table 5. Comparison of Hedione isomers as modulators ........................... 39
Table 6. Schemes for odor delivery ............................................................. 98
Acknowledgements

My deepest gratitude goes to my advisor, Prof. Stuart Firestein, for all the guidance, support, trust and freedom he granted me over the past many years. From him I have not only learned how to be a better scientist, but also how to be a bigger person. I feel extremely fortunate and proud to be part of his lab, and I could not have imagined to have a better advisor.

I would also like to thank all current and former Firestein lab members, especially the ones who have paved the way for me. Dr. Zita Peterlin coached me for the first two years in her instructive and artistic way, introducing me to the world of olfaction and medicinal chemistry. Dr. Dong-Jing Zou enlightened me with his encyclopedic knowledge and provided valued advice on thesis writing. Our lab manager Cen Zhang helped me a great deal with animal breeding and genotyping. She took care of everything and everyone, making the lab feel like a family. I also want to thank my former labmates and friends, Drs. Jessica Brann, Erwan Poivet, Narmin Tahiroya, Ann-Sophie Barwich and many others, for their stimulating discussions and encouragement that helped me through the most difficult time.

Outside of the Firestein lab, I want to express my sincere gratitude to Prof. Elizabeth Hillman, who generously shared her expertise in optical imaging and granted me the freedom of utilizing all kinds of resources in her lab. I also want to thank all the Hillman lab members for their help and support.

I would like to thank my thesis committee members, Profs. Darcy Kelley, Jian Yang and Stavros Lomvardas, for their insightful advice. I want to thank Prof. Aurel Lazar, who sponsored my first rotation. My gratitude also goes to Profs. Haiqing Zhao, Brian McCabe, Drs. Yevgeniy
Slutskiy, Christopher Ferguson and Master Perfumer Christophe Laudamiel, for their guidance and expertise at critical points.

My special thanks goes to Dr. Wenze Li, for being both a compassionate spouse and an intelligent collaborator; and our dog Muffin, for being my psychotherapist and muse.

Last but not the least, I would like to thank my parents, Ya-Qiong Wang and Wen-Lin Xu, for their unwavering support and unconditional love.
1.1. Anatomical and molecular basis of the peripheral olfactory system

1.1.1. Functional anatomy of the olfactory epithelium

The olfactory system is a critical component of the three chemosensory systems, the other two being the gustatory and the trigeminal systems. It is not only responsible for sensing odors, but also participates in flavor perception and chemically mediated aesthetic perceptions [1]. Most mammals and reptiles have two segregated olfactory systems: a main olfactory system, which detects regular odors and some pheromones through the main olfactory epithelium; and an accessory olfactory system, which detects pheromones through the vomeronasal organ (VNO). This thesis focuses primarily on the main olfactory system.

In vertebrates, odor sensing begins from the olfactory epithelium (OE), a pseudostratified columnar epithelial tissue. Human OE measures 2-10cm² in total, covering the cribriform plate, the superior septum, as well as the superior and middle turbinates [2-4]; the distribution of mouse OE is similar but shifted more ventrally to cover a larger portion of the septum and turbinates.
The OE consists of several different types of cells, each playing a critical role in maintaining the normal function of the epithelium. The olfactory sensory neurons are responsible for sensing odors; supporting cells provide metabolic and physical support for the epithelium; basal cells are stem cells capable of differentiating into olfactory or supporting cells; Bowman's glands secrete proteinaceous substance that forms the mucus layer (Figure 1) [5]. Among these cells, the olfactory sensory neurons (OSNs) play the most important role in olfactory sensing. OSNs are bipolar neurons, each neuron has 10-30 cilia emanating from the dendritic knob onto the overlying mucus layer (produced by the Bowman’s gland) to detect airborne volatile molecules, and an axon projecting back through the cribriform plate to the olfactory bulb [6]. The cilia are about 30μm in length and 0.1–0.3μm in diameter, enmeshed with each other, providing an extensive surface area for odor detection [3, 7]. The membranes of these cilia are enriched with odorant receptors (ORs), which directly interact with airborne chemicals through ligand-receptor binding [8].

Figure 1. Structure of the olfactory epithelium. The major cell types are illustrated, including mature and immature olfactory sensory neurons, supporting cells, basal cells and Bowman's glands. (Adapted from NEUROSCIENCE, fourth edition)
OSNs are generated throughout life in the olfactory epithelium. Specifically, a sub-population of basal cells gives rise to neuronally-committed $Ascl1^+$ progenitors and subsequently $Neurog1^+/Neurod1^+$ immediate neuronal precursors, which then generate $Cxcr4^+/Dbn1^+$ nascent OSNs. The nascent OSNs rapidly transition into $Gap43^+$ immature OSNs, which eventually become mature OSNs [9, 10]. Full maturation of OSNs is marked by the expression of olfactory marker protein ($Omp$) [11].

1.1.2. Odorant receptors and the one-neuron-one-receptor rule

The odorant receptor (OR) gene family was first identified in 1991 [12]. It contains ~800 members (including ~400 pseudogenes) in human and ~1400 (including ~200 pseudogenes) in mouse [13-15], comprising one of the largest gene families in mammals. While immature OSNs may transiently express more than one receptor [16-18], each mature OSN only expresses one allele of one OR gene [19-21]. Although the underlying mechanism remains unclear, this ‘one-neuron-one-receptor’ rule has been directly or indirectly validated by several lines of experiments, including single-cell RT-PCR on limiting-diluted OSNs [19], dual-probe in situ hybridization or gene labeling of OSNs and glomeruli [22-25], functional analysis of OSN responsiveness [26, 27], and next generation sequencing of single-cell transcriptome [16, 17, 28]. A few exceptions have been reported, but the mRNA transcript expression levels of the co-expressed ORs were much lower (usually by three magnitudes) than the dominant OR; some of them turned out to be pseudogenes [16-18, 22, 28]. Therefore, the odor response of a single mature neuron also represents the activity of the receptor it expresses. This feature allows us to characterize the populational response profile of receptors through high-throughput recording of neurons without knowing the genetic identity.
of each receptor. The axons of OSNs expressing the same receptor converge the ipsilateral half of the olfactory bulb, forming one or more dense neuropils termed glomeruli [29-31].

1.1.3. OR classification and zonal expression

Phylogenetic analysis indicated that the ORs can be divided into two classes: class I (fish-like) ORs that sense water-soluble odors, and class II (mammalian-like) ORs that sense volatile odors [32-34]. Teleost fish have only class I OR genes; amphibians express both class I and class II ORs [35]. While mammalian OR genes are mostly class II ORs, class I ORs also account for 10-20% of the entire OR repertoire, indicating an indispensable role of class I ORs in mammalian olfaction [34, 36, 37].

Historically, the murine olfactory epithelium was divided into four parallel zones (Zone 1-Zone 4), each harboring its own subset of ORs [38-40]. Zone 1 is localized in the dorsomedial region, consisted of most class I ORs and some of the class II ORs [34, 41]; Zone 2-4 are localized in the ventrolateral region, where the majority of class II ORs are expressed [34, 42]. This dorsomedial-ventrolateral difference is also reflected by non-OR gene expression: in particular, Acsm4 (also known as O-MACS) [43] and Nqo1 [44] are markers for the dorsomedial region (Zone 1) and Ncam2 (also known as Ocam) for ventrolateral (Zone 2-4) [45]. Aside from the well-defined segregation between Zone 1 and Zone 2, there seems to be no clear boundary between other zones; instead, expression regions are OR-specific, distributed along the dorsal-ventral axis in an overlapping and continuous manner [42, 46, 47]. In addition to the regular strip-like expression pattern, some ORs exhibit ‘unusual’ expression patterns and form more isolated and restricted patches, such as MOR120-1 [48] and the MOR262 family [49].
A recent study indicated that the zonal organization in the olfactory epithelium might be more complex than previously thought. In this study, the expression patterns of 68 OR genes were probed with multiplex three-color fluorescence in situ hybridization and mapped to the 3D-reconstructed mouse olfactory epithelium. The 68 ORs revealed nine overlapping and complex zones (Figure 2); however, given the large size of the mouse OR gene repertoire (>1,000), it would not be surprising to find additional zones [50].

Figure 2. Zonal expression of odor receptors in mouse. The expression areas of 68 OR genes were classified into 9 zones, including Zolf160, Zolf24, Zolf17, Zolf155, Zolf374, Zolf2, Zolf1507, Zolf15 and Zolf653. Only seven Zolf zones are shown because Zolf15 and Zolf653 are not visible on this schematized section. Abbreviations: D, dorsal; V, ventral; M, medial; L, lateral. (Adapted from Zapiec and Mombaerts, 2020)
1.1.4. OSN signaling transduction

Most mammalian ORs are class A G-protein-coupled receptors (GPCRs). Typically, the signaling cascade is initiated by odor molecules (ligands) binding to the receptor on the ciliary surface of mature OSNs (Figure 3). The receptor then recruits the olfactory-specific heterotrimeric G-protein (Goif) [51], which activates an olfactory-specific adenylate cyclase (ACIII) [52]. ACIII converts ATP into cyclic AMP (cAMP), the latter opens the cyclic nucleotide-gated (CNG) cation channels [53]. Subsequent Na\(^+\) and Ca\(^{2+}\) influx and membrane depolarization further activates the calcium-activated chloride channels (CaCCs), causing an outward flowing and depolarizing chloride current [54, 55]. The calcium rise in the lumen of the OSN cilia also triggers intracellular calcium release, leading to prolonged calcium responses in dendrite and soma [56].

**Figure 3. Signal transduction in mammalian OSNs.** Abbreviations: OR, odorant receptor; ACIII, adenylate cyclase III; CNGC, cyclic nucleotide-gated channel; CaCC, calcium-activated chloride channel. (Adapted from Kaupp, 2010)

Odor-induced adaptation happens at different signaling stages and time scales. Fast odor adaptation occurs within seconds, primarily mediated by calcium influx through CNG channels. In this process, Ca\(^{2+}\) ions rapidly bind to apocalmodulin (the calcium-free version of calmodulin) to form the Ca\(^{2+}\)/calmodulin complex, which suppresses CNG channel activity by decreasing its
binding affinity for cAMP [57, 58]. In addition, Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) attenuates ACIII activity through phosphorylation, causing desensitization to prolonged odor stimulus [59, 60]. The Ca\(^{2+}\)/calmodulin complex also activates phosphodiesterase (PDE) that stimulates the hydrolysis of cAMP. Two types of PDEs have been found in OSNs: PDE1C, which localized in the cilia and PDE4A in the rest of OSN but not in the cilia [61, 62]. Regardless of the discrete cellular distribution, these two PDEs seem to be functionally redundant to each other [63].

Ca\(^{2+}\)-independent mechanisms also contribute to odor adaptation. For example, the carbon monoxide/cyclic guanosine-monophosphate (CO/cGMP) second messenger system mediates the long-lasting adaptation, a slow form of adaptation that operates on a time scale of minutes [64]. Odor adaptation could also be induced through phosphorylation of GPCRs, which involves the participation of various signaling factors, such as cAMP-dependent protein kinase (PKA), G protein-coupled receptor kinase 3 (GRK3, also known as β-adrenergic receptor kinase 2) and β-arrestin2 [65-68].

The recovery of the odor-sensing machinery to the resting state is achieved by extrusion of ciliary Ca\(^{2+}\) and closing of CaCCs. The cytoplasmic Ca\(^{2+}\) is removed by Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX), potassium-dependent Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCKX4) and Ca\(^{2+}\)-ATPase [69-72]. Meanwhile, the chloride gradient is restored by the Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) cotransporter (NKCC1) [73]. This resetting mechanism enables us to stimulate OSNs with a sequence of odors in vitro so long as a 3-5min interval is allowed for recovery from each stimulus.

Some OSNs sense odors through non-canonical signaling pathways. For example, guanylyl cyclase (GC-D)-expressing neurons use the cGMP signaling pathway [74], a pathway that is also utilized by the Grueneberg Ganglion (GG) neurons [75]. In addition, a subset of GC-D neurons (necklace sensory neurons) express a group of non-canonical odorant receptors named MS4As,
which are four-transmembrane domain proteins. Unlike canonical ORs, multiple MS4A genes are co-expressed in a single neuron [76]. The complete signaling machinery of these MS4A-expressing neurons is not clear yet; one study suggested that PLCγ1 and Orai1 might be involved [77]. Another example is Olfr73-expressing OSNs, which were shown to have an alternate signaling pathway mediated by Gnao [78]. On the other hand, some non-canonical odorant receptors are still coupled to the canonical cAMP signaling components, such as trace amine-associated receptors (TAARs) [79].

It has long been speculated that the Inositol-trisphosphate (IP3) pathway may also contribute to odor sensing [80-83]. IP3 has been shown to mediate olfactory sensing in some insects [84-86], and may play a modulatory role in mammalian olfactory sensing [87]. However, knockout of components of the cAMP pathway usually resulted in anosmia in mice [51-53]. Some researchers reported that odor responsiveness was partially preserved in Cnga2 knockout mice, in which case phospholipase C might be involved [88]. This residual odor responsiveness might also come from the downstream activation of transient receptor potential channel M5 (TRPM5) channels, which are co-expressed with CNGA2 in a subset of OSNs [89-91].

1.2. Encoding of olfactory information

To date, how to make sense of a smell remains a mystery. Although the mechanism of vision has been well understood and leveraged to perform many challenging tasks (e.g., computer vision), several discrepancies between modalities have prevented this knowledge from being extrapolated to olfaction. The first difference is input complexity. The visual system detects photons, which can be uniformly described by one parameter (wavelength). The olfactory system, in contrast, detects odor molecules, which are highly diverse in their properties. The difference in stimulus complexity
is also reflected by the number of receptors in both systems (3 vs. 1000). Second, the visual processing pathway is highly organized with a topographic map preserved even in the higher processing centers, whereas the organization of olfactory pathway seems to be obscure. Neural tracing experiments have shown that projection from olfactory bulb to piriform cortex is highly distributive without discernable topographic arrangement [92-94]. Finally, the outputs are also different. The attributes of visual perception (such as color, orientation and contrast) are continuums, while odor qualities seem to be sparsely arranged in space with unknown dimensionality and primacies. For example, it is difficult to imagine an intermediate smell between rose and potato chips. In summary, the visual system tends to constructively convert low-dimensional stimulus to high-dimensional attributes, whereas the olfactory system seems to convert the high-dimensional input to relatively low-dimensional perception. Therefore, the underlying mechanism of olfactory information processing might be without current precedent, and a better understanding of it would potentially benefit the development of artificial intelligence and many other inter-disciplinary fields.

1.2.1. Navigating the odor space

Historically, many attempts have been made to find the primacies of the odor space. A pioneering model was Henning’s smell prism, in which six primary odor qualities (Flowery, Foul, Fruity, Spicy, Burnt and Resinous) occupy the corners of a triangular prism, while other odors being distributed within this three-dimensional space (Figure 4) [95]. In Crocker and Henderson’s odor classification system, odors were numerically evaluated with four attributes (Fragrant, Acid, Burnt and Caprylic) [96]. In 2002, McGinley and McGinley proposed an “odor wheel” diagram, in which odor qualities were divided into eight categories - Vegetable, Fruity, Floral, Medicinal, Chemical,
Fishy, Offensive, and Earthy [97]. Later in 2015, Zarzo developed a 3D sensory map (odor cube) based on statistical analysis of numeric odor profiles, and quantitatively interpreted the relationship between several frequently used descriptors in perfumery (Figure 5) [98]. Overall, these verbal-based coordinate systems share many features in common and are to some extent interchangeable, which is expected in a vectorized space. However, there is no consensus on the number and identity of odor primacies, let alone the proof of their orthogonality.

![Figure 4. Henning’s smell prism. (Adapted from Henning, 1916)](image-url)
Figure 5. Zarzo’s odor cube showing common perfumery descriptors. Colored areas highlight the four major attributes (Fragrant, Acid, Burnt and Caprylic) in Crocker and Henderson’s classification system. Pungent, Garlic, Camphoraceous and Putrid descriptors cannot be properly placed in the cube and are indicated with dashed arrows. (Adapted from Zarzo, 2015)

The development of data mining algorithms has facilitated the navigation of perceptual space with physicochemical properties available in databases [99]. To simplify the chemical-perception model, Khan et al. reduced the dimensionality of odor perception through principle component analysis (PCA) of 146 verbal descriptors, and identified the first principle component (PC1) as pleasantness [100]. This primary perceptual axis was later utilized to predict the hedonic valence of novel odors with >80% similarity to human perception [101], yet the abundance of information was limited by the single output parameter. Recent advances in machine learning and neural network have enabled researchers to build more complex models: based on the perceptual profile of 476 odor molecules reported by 49 individuals and 4,884 physicochemical properties,
Keller et al. trained models to predict odor qualities (8 of 19 semantic descriptors) as well as odor intensity and pleasantness [102]. The predictive accuracy was not optimal, partially due to the relatively small dataset compared with the normal size of dataset used for deep learning. Nevertheless, this study revealed several interesting features. For example, among the 19 descriptors, “garlic/fish”, “musky/sweaty”, and “sweet/bakery” were found to be positively correlated pairs; prediction of “bakery” was driven by molecular similarity to vanillin. In summary, it is theoretically possible to predict the averaged human perception directly from the chemical properties of odor compounds, but the complexity of both the input and the output on top of the requirement of a large enough training dataset has tremendously increased the difficulty of this task. More understanding of the biological basis of odor encoding is thus demanded to assist the development of such algorithms.

1.2.2. The molecular receptive range of ORs

The identification of odorant receptor genes has largely bridged the gap between odor stimulus and perception [12]. Following this milestone work, a series of studies have been conducted to characterize the relationship between odors and receptors. It is now widely appreciated that each receptor recognizes multiple odors and each odor is recognized by a variety of ORs [103]. Studies of a deorphanized receptor, OR-I7, revealed several rules underlying ligand-receptor binding [104-106]. The first rule is that if one OR responds to both \( n \)- and \((n+2)\)-carbon compounds, it will also accept the intermediate form with \( n+1 \) carbons; the second rule is that if one OR responds to both an \( n \)-alcohol and its homologous \( n \)-acid, it will also accept the homologous \( n \)-aldehyde. These rules, along with many other observations, are consistent with many concepts in medicinal chemistry. In medicinal chemistry, fragments in chemicals are frequently replaced to achieve desired biological functions (receptor activity in this case). Following the same strategy, more
rules were then discovered through studies of benzene and ester derivatives. One is that if one OR responds to both a benzene derivative and a homologous furan compound, it will also accept the homologous thiophene compound, which bears intermediate topological polar surface area (TPSA) [107]. Another is that ORs tolerate ester reversal better than ester displacement in the backbone [108]. These findings indicated the importance of topology and electronegativity in odor-receptor binding, providing valuable reference when predicting odor responses from chemical properties.

1.2.3. The complexity of olfactory coding

The generally appreciated combinatorial olfactory coding was based on interactions between individual receptors and odors. However, the olfactory world is complex, and almost all naturally-occurring smells are odor mixtures. For example, the fragrance of rose is composed of rose oxides (\textit{cis}-rose oxide and \textit{trans}-rose oxide), rose ketones (\textit{\beta}-damascenone, \textit{\beta}-damascone, \textit{\beta}-ionone), terpene compounds (geraniol, nerol, citronellol) and many other volatile chemicals [109]. Notably, rose oxides and rose ketones are only present in minimal amounts, but strongly contribute to the note of roses.

The perceptual difference between odor mixtures and their individual components has been frequently observed in psychophysical experiments [110-114]. More specifically, the intensity of an odor mixture is often found to be less than the summed intensity of its constituents. The underlying mechanism of this phenomenon is unclear. It can be partially explained by information compressing or psychophysical scaling [115, 116]; however, these general information processing steps are not sufficient to explain those “outlier” odor pairs that seemed to be more suppressive than others [110, 112].
Though not as extensively studied as in psychophysics, modulated responses to odor mixtures in the periphery has also been occasionally reported. For example, citral was found to be an antagonist of octanal at the OR-I7 receptor [104], and methyl isoeugenol an antagonist of eugenol at the mOR-EG receptor [117]. While these two cases of antagonism between odors can be attributed to the high structural similarity within each odor pair, another study showed interaction between two chemically unrelated odors, whiskey lactone and isoamyl acetate [118]. Interestingly, an earlier research showed that odor-evoked OSN responses could also be inhibited by adrenergic and muscarinic antagonists, suggesting a wide spectrum of possible antagonists [119]. However, most experiments on the odor interactions were performed in heterologous OR-expression systems or with a small number of individual olfactory neurons due to technical limitations.

To gain a more comprehensive understanding of odor interactions in mixtures, I developed an OSN imaging protocol utilizing a novel microscopy technique, Swept Confocally Aligned Planar Excitation (SCAPE) for OSN imaging with my collaborators from the Hillman Lab. The novel technique enabled us to record the calcium signal of over 10,000 olfactory sensory neurons in the olfactory epithelium of a single mouse. Eight chemically distinct odors were tested individually or in odor blends of up to 3 odors. As a result, we observed diverse modulation events, including antagonism, partial agonism and enhancement. Our result shows that, in contrast to the visual and other sensory systems, olfactory information is subject to modulation at the primary sensing level, probably at a higher frequency than one would normally expect. Further experiments are needed to evaluate the psychophysics outcome of peripheral odor modulation.
Chapter 2

THE MEDICINAL CHEMISTRY PRINCIPLES BEHIND PERIPHERAL ENCODING OF FLORAL ODORS

2.1. Introduction to medicinal chemistry

Predicting odor quality from the chemical properties of a given compound remains a challenge today. One obstacle is that odorant receptors do not necessarily follow the same logic of the conventional organic chemistry scheme in recognizing odor chemicals. For example, the two-carbon difference between γ-nonalactone and γ-undecalactone is sufficient to shift their notes from coconut to peach, whereas β-damascone and (-)-cis-rose oxide do not show apparent structural similarity, but both smell rosy.

Fortunately, linking chemical properties with biological activity can be facilitated by medicinal chemistry. Medicinal chemistry emphasizes bioactivities over chemical properties, and evaluates the function of chemical subgroups based on the biological outcome of fragment
substitution. If the biological property of the derived compound is similar to the original compound, they are bioisosteres of each other and this phenomenon is termed bioisosterism.

In this project, seven structurally-related odor compounds were selected and assigned to three groups, each reflecting a different type of fragment substitution. The biological activity of all seven compounds were evaluated and compared based on their OSN response profiles. Hierarchical clustering analysis showed that fragment substitution led to diversified biological outcomes, possibly depending on the chemical and biological context.

2.2. Experiment Design

Seven odor compounds were selected for this study: coranol (COR), carbinol muguet (CM), nerol (NER), geraniol (GER), mayol (MAY), β-ionone (bION) and β-damascone (bDAM). All compounds are commonly used odors in perfumery and impart a variety of floral notes. Their chemical structures and perceptual descriptions are listed in Table 1.

These floral odors can be further classified into three panels. Panel A consists of COR and CM, in which the alkyl ring (cyclohexane) of COR is replaced by an aromatic (benzene) ring in CM. Panel B consists of NER, GER and MAY, all bearing a terpene-like scaffold. While the conformations of NER/GER are more flexible thanks to the rotatable C4 and C5 bonds, the conformation of MAY is restricted by its cyclohexane ring. Panel C consists of bION and bDAM. These two odors differ only in the orientation of the 3-penten-2-one arm, yet impart distinctive odor qualities (violet vs. rosy). The three groups of odors therefore represent three different types of bioisosteric substitution: ring replacement, backbone lock, and fragment reverse.
<table>
<thead>
<tr>
<th>Panel A</th>
<th>Chemical name (abbrev.) and CAS#</th>
<th>Chemical structure</th>
<th>Odor description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coranol (COR) 83926-73-2</td>
<td></td>
<td>floral, rosewood, coriander</td>
<td></td>
</tr>
<tr>
<td>Carbinol muguet (CM) 103-05-9</td>
<td></td>
<td>clean, fresh, floral-rose and violet</td>
<td></td>
</tr>
<tr>
<td>Panel B</td>
<td>Chemical name (abbrev.) and CAS#</td>
<td>Chemical structure</td>
<td>Odor description</td>
</tr>
<tr>
<td>Nerol (NER) 160-25-2</td>
<td></td>
<td>sweet, floral-neroli, magnolia, fruity-citrus</td>
<td></td>
</tr>
<tr>
<td>Geraniol (GER) 106-24-1</td>
<td></td>
<td>sweet, floral-rose, fruity-citrus, peach, waxy</td>
<td></td>
</tr>
<tr>
<td>Mayol (MAY) 5502-75-0</td>
<td></td>
<td>fresh, clean, floral-magnolia, hydroxycitronellal, cumin, grassy</td>
<td></td>
</tr>
<tr>
<td>Panel C</td>
<td>Chemical name (abbrev.) and CAS#</td>
<td>Chemical structure</td>
<td>Odor description</td>
</tr>
<tr>
<td>β-ionone (bION) 14901-07-6</td>
<td></td>
<td>floral-violet, woody, sweet, fruity-raspberry, green</td>
<td></td>
</tr>
<tr>
<td>β-damascone (bDAM) 35044-68-9</td>
<td></td>
<td>floral-rose, fruity-blackcurrant, plum, honey, tobacco</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. *Floral panel compounds.* Color shading indicates compound panels A, B and C.

2.3. Methods

2.3.1. Chemicals

All seven odors were gifts from Firmenich SA. Odors were first dissolved in >99% dimethyl sulfoxide (DMSO, Sigma-Aldrich) into 30mM stocks, then diluted 1:1000 in freshly prepared Ringer’s solution (see Table 2) to a final concentration of 30μM before experiment.
2.3.2. Animals and tissue collection

All animal procedures complied with Columbia University’s guidelines for care and use of animals. Male C57BL/6J (JAX000664) mice at 6-8 weeks of age were overdosed with anesthetics (ketamine, 90mg/kg; xylazine, 10mg/kg, i.p.) and decapitated. The head was cut open sagittally and the septum was removed to expose the medial surface of the olfactory turbinates in cold divalent-free Ringer’s solution. The olfactory epithelium was collected from the septum, the dorsal recess and the turbinates, cut into small pieces and incubated in 5ml of digest solution (see Table 2) at 37°C for 45min. The digested tissue was then washed with 10mL of culture medium (see Table 2) and transferred into a clean 15mL conical tube containing 50μL of culture medium. To dissociate OSNs from epithelium, the conical tube was tapped sharply against a hard surface. The supernatant culture medium containing OSNs was drawn and split onto four concanavalin A (Sigma-Aldrich, 10mg/mL)-coated glass coverslips, each plated in 35mm Petri dishes. After allowing the cells to settle for 15min, each dish was filled with 2mL of culture medium and placed in a cell incubator at 32°C for more than 1h. The recipes of all solutions used in this project are listed below (Table 2).

<table>
<thead>
<tr>
<th>Solution name</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>i Ringer’s solution</td>
<td>mM: 138 NaCl, 5 KCl, 1 CaCl₂, 1.5 MgCl₂, 10 Hepes, 10 d-glucose, pH 7.4</td>
</tr>
<tr>
<td>ii Ringer’s solution (divalent-free)</td>
<td>mM: 145 NaCl, 5.6 KCl, 10 Hepes, 10 glucose, 4 EGTA, pH 7.4</td>
</tr>
<tr>
<td>iii Digest solution</td>
<td>5mg/ml bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), 0.5mg/ml collagenase, 22U/ml dispase (Gibco BRL, Grand Island, NY, USA) and 50μg/ml deoxyribonuclease II (Sigma), dissolved in divalent-free Ringer solution to a final volume of 5mL.</td>
</tr>
<tr>
<td>iv Culture medium</td>
<td>DMEM/F12 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1x insulin-transferrin-selenium (Gibco BRL), 100U/ml penicillin, 100μg/ml streptomycin (Gibco BRL) and 100μM ascorbic acid (Sigma-Aldrich).</td>
</tr>
</tbody>
</table>

Table 2. Solutions for acute OSN culture preparation.
2.3.3. Calcium imaging

Calcium imaging recordings were performed as described in detail elsewhere [104]. Briefly, the OSN-coated coverslip was rinsed with Ringer’s solution and loaded with Fura-2AM (5μM) plus pluronic acid F127 (80μg/ml; Molecular Probes, Eugene, OR, USA) for 45min at room temperature. The coverslip was then mounted onto a recording chamber and imaged at room temperature under 380nm excitation and 510nm emission light using the NIH Image software (NIH, Bethesda, MD, USA). Images were acquired every 4s, each image was averaged from three frames. During the image session, cells were continuously perfused with Ringer’s solution at 0.75mL/min. Odor stimuli were delivered by injecting 400μL of the odor solution into the chamber over 4s through a custom-made manifold tubing system, with a 2min interval between stimuli. In each experiment, the odor sequence was pseudo-randomized within and across panels. Data presented in Figure 7 were re-ordered for display purpose. At the end of each experiment, the adenylate cyclase activator forskolin (50μM) was applied to verify the functional viability of all OSNs.

2.3.4. Data analysis

Data were shown as ΔF/F₀, where ΔF is the fluorescent light intensity change at each data point and F₀ is the baseline. Responses were measured as peak ΔF/F₀ change. To account for rundown, odor responses were linearly corrected using the two flanking odor stimuli presented in each panel. Previous work from our lab has shown the variance of responses to the same odor stimulus to be less than 10% [120]. If the cell did not respond to the flanking odor, response in between will not be corrected.
2.4. Results

About 900 forskolin-responsive cells were yielded from three experiments (2 mice were used in each experiment). Among these cells, 108 OSNs that were responsive to the floral odors were utilized for further analysis. An example of single cell time course can be seen in Figure 6.

![Figure 6. Time course of an individual OSN.](image)

Peak responses were measured and normalized to the maximum odor response of each OSN. The result was plotted as a heatmap, where each row represents a neuron and each column represents an odor stimulus (Figure 7A). Interestingly, some OSNs (5 out of 108) seemed to be widely tuned and were activated by all seven compounds. Response-based odor similarity was then evaluated with hierarchical clustering (Figure 7B).
Figure 7. Floral panel data summary. (A) Heatmap of normalized peak responses (N = 108, 6 mice). Each row represents an individual OSN and each column represents an odor stimulus. Responses were normalized to the highest odor response of each OSN. (B) Response-based hierarchical clustering of floral odors. The euclidean distances between clusters were calculated from normalized peak responses of each OSN.

Several features can be extracted from the clustering result. First, COR and CM turned out to be the closest odor pair across all seven odors. It is a bit unexpected that the neuronal response profile of COR and CM are more similar than those of NER and GER, given that NER and GER only differ in their isomeric conformations. It thus suggested that aliphatic and aromatic rings might be interchangeable under certain circumstances. Second, MAY was clustered close to NER and GER. In addition, the response profile of MAY seemed to overlap more with that of GER than NER (Figure 7A), probably because of their higher similarity in molecular conformation, especially the relative position of the isobutyl(ene) tail and the hydroxyl group. Finally, bION and bDAM were the most distant pair of odors within and across the three panels. This result is
consistent with their distinctive odor qualities. One explanation is that reversing the 3-penten-2-one arm causes electron cloud re-distribution and polarity change, which may play a critical role in odor-receptor binding.

2.5. Discussion

This is my first project since I joined the Firestein Lab in Spring 2013. It was designed and conducted with the guidance of Dr. Zita Peterlin, as a proof-of-concept study of using medicinal chemistry to understand odor-receptor relationship in the olfactory system. The selection of the seven compounds that are commonly used in perfumery allowed us to explore bioisosterism with the help of background knowledge in perception. This study showed that chemically distinctive odors, such as COR and CM or GER and MAY, can be bioisosteres, whereas odors with similar molecular formula, such as bION and bDAM, can recruit different subset of receptors. However, since only seven odors were utilized in this research, and each type of substitution was only tested across two or three odors, it was not sufficient for a comprehensive interpretation of the biological outcome of these changes.

In two follow-up studies conducted together with Dr. Erwan Poivet et al., we investigated the coding of heterocyclic compounds and esters, respectively, with an expanded number of chemical derivatives in each panel [107, 108]. In these projects, I performed odor classification with Matlab scripts, some results are shown below (Figure 8-9). Applying this medicinal chemistry approach, we were able to link biological responses with molecular properties and identify molecular descriptors critical for peripheral odor discrimination, such as topological polar surface area (TPSA) and a few other electronegativity-related indices. Some of these descriptors are listed in Table 3.
Figure 8. Hierarchical clustering analysis result of the heterocyclic compound panel. The Euclidean distance between each odor pair was calculated based on odor-elicited neuronal responses from 276 OSNs. (For more details, see Poivet et al., 2016).
Figure 9. Hierarchical clustering analysis result of the ester panel. The Euclidean distance between each odor pair was calculated based on odor-elicited neuronal responses from 872 OSNs. (For more details, see Poivet et al., 2018).
Table 3. Top 20 e-Dragon descriptors of odor discrimination in the ester panel. Each of the 1,666 molecular descriptors from the e-Dragon database was cross-compared with the OSN response profile to find the ones that best recapitulate the biology-based clustering result. The top 20-ranked descriptors and their Spearman’s correlation factor (rho) are shown above. (For more details, see Poivet et al., 2018).
Chapter 3

EXPLORING THE MODULATION EFFECTS OF HEDIONE AND HEDIONE-LIKE MODULATORS

3.1. Introduction to the Hedione effect

The legend and mystery of Hedione has persisted for more than 60 years. Hedione, or methyl dihydrojasmonate, was first synthesized as a replacement of methyl jasmonate, a key component in the smell of jasmine. This turned out to be a failure, as the smell of Hedione was too subtle on its own. Nevertheless, perfumers found it to be a versatile enhancer that made fragrances (especially citrus and floral odors) “fresher and radiant”. Hedione has therefore become, and remains, one of the most important and widely used ingredients in perfumery.

Despite the abundant perfumery knowledge about the modulation effect of Hedione, its molecular mechanism has remained largely unknown. In this study, I aimed to explore the modulation effects of Hedione from multiple perspectives, including modulation spectrum, binding affinity and molecular features keyed to the modulation effects.
3.2. Methods

3.2.1. Animals and tissue collection

All animal procedures complied with Columbia University’s guidelines for care and use of animals. OMP-Cre\(^{+/−}\) ROSA-GCaMP3\(^{+/−}\) mouse strain used in this work was generated by crossing the OMP-Cre line (JAX 006668) with the Ai38 line (RCL-GCaMP3, JAX014538). In these compound mutant mice, the expression of the genetically encoded calcium sensor GCaMP3 is restricted to the mature olfactory sensory neurons. Compared with the Fura-2 loading method described in the previous chapter, GCaMP3 offers better SNR and faster kinetics, and better specificity (mature OSN only). In addition, since GCaMP3 is a genetic tool, it causes less physical damage to neurons than a dye loading procedure.

Mice used for experiments were males at 6-8 weeks of age. Acute primary culture of OSNs was prepared as described previously (see Chapter 2 Methods). Briefly, the olfactory epithelium was dissected out from the septum and turbinates in cold divalent-free Ringer’s solution. After tissue digestion, OSNs were dissociated from the epithelium and coated on a glass coverslip, which was then mounted on a recording chamber for imaging.

3.2.2. Calcium imaging

Calcium imaging was performed on an inverted fluorescence microscope (IMT-Olympus, Tokyo, Japan) equipped with a SIT camera (C10600, Hamamatsu Photonics, Hamamatsu, Japan), a Lambda XL light source (Sutter Instrument, Novato, CA, USA), and Lambda-10B optical filter changer (Sutter Instrument). Samples were imaged under 490nm excitation and 520 nm emission
light. Image series were acquired every 4s with Metamorph Premier software (Molecular Device LLC, Downingtown, PA, USA).

During the image session, cells were continuously perfused with Ringer’s solution at 0.75mL/min with a 1260 Infinity HPLC isocratic pump (Agilent Technologies, Santa Clara, CA, USA). Odor stimuli were presented by injecting 100μL of the odor solution into the perfusion chamber through a 1260 Infinity HPLC autosampler (Agilent Technologies, Santa Clara, CA, USA) at 200μL/min, with a 3.5min interval in between. At the end of each experiment, the adenylate cyclase activator forskolin (50μM) was applied to verify the functional viability of all OSNs. The images were then processed with Metamorph Premier software to extract single cell time courses.

3.2.3. Data analysis

Single cell time courses were extracted from image series using Metamorph Premier software. Data were calculated as $\Delta F/F_0$, where $\Delta F$ is the fluorescent light intensity change at each data point and $F_0$ is the baseline. Responses were measured as peak $\Delta F/F_0$ change.

When measuring the modulation effects of Hedione and other modulators, a modulation index ($I_{\text{mod}}$) was calculated as $I_{\text{mod}} = (d_1-d_0)/d_0$, where $d_0$ represents response to the leading odor X, $d_1$ represents response to the binary mixture of X and the modulator. Typically, odor stimuli were presented in a sequence of (X, X + modulator, X); accordingly, $d_0$ was calculated as the averaged response of the two flanking X.
3.3. Results

3.3.1. Probing the modulation spectrum of Hedione

Based on psychophysical experience in perfumery, Hedione shows more apparent effects on floral and citrus odors. To gain a better understanding of how and to what extent Hedione (HED) could modulate other odors, I tested the effect of 100μM HED on four floral/citrus odors that are commonly used in perfumery: citral (CIT), mayol (MAY), eugenol (EUG) and vanillin (VAN), each at 30μM. The sequence of odor stimuli is shown in Figure 10.

Figure 10. Time courses of two individual OSNs showing the modulation effects of Hedione. Neurons were stimulated with 30μM of citral (CIT)/mayol (MAY)/eugenol (EUG)/vanillin (VAN), with or without 100μM Hedione (HED). One neuron (top) showed responses to CIT and MAY, both inhibited by HED; the other (bottom) showed an enhanced response to EUG by 100μM HED.

87 OSNs were activated by the four floral/citrus odors or their mixtures with HED and not activated by HED alone. To quantify the modulation effects, a modulation index ($I_{mod}$) was calculated as $(d_1-d_0)/d_0$, where $d_0$ represents the averaged response to the leading compound alone.
and \( d_1 \) represents the response to the mixture of this compound plus 100\( \mu \)M HED. Here, \( I_{\text{mod}} < -0.3 \) indicates inhibition, \( I_{\text{mod}} > 0.3 \) indicates enhancement, while a value between -0.3 and 0.3 is conservatively considered to be within variance. The results are summarized as a heatmap in Figure 11. Both enhancement and inhibition were observed with all four odors, suggesting the modulation by HED is widespread. In particular, responses to CIT were more frequently inhibited than enhanced, while responses to VAN were more enhanced than inhibited.

**Figure 11. Diversified modulation effects of Hedione on floral odors.** OSNs were stimulated with 30\( \mu \)M of CIT/MAY/EUG/VAN, with or without 100\( \mu \)M HED. The modulation effects were quantified with the modulation index (\( I_{\text{mod}} \)) and shown as a heatmap. Each row represents a single OSN; each column represents the modulation index of the corresponding odor. Red indicates inhibition, blue indicates enhancement. Color transparency indicates the normalized response intensity of each neuron. \((N = 87)\)
3.3.2. **Dose-response analyses of the Hedione effect**

The predominance of Hedione’s inhibitory effect on CIT responses was counter-intuitive, as CIT is known to be psychophysically “enhanced” by HED. To further investigate the mechanism of inhibition, I performed a series of dose-dependent experiments on the CIT-HED binary pair. In a preliminary CIT dose-response experiment, the RC$_{50}$ (the concentration at which 50% of CIT-responsive neurons are recruited) of CIT was determined to be 34.6μM, while the saturating concentration was ~1000μM (N = 44; see Figure 12).

![Figure 12. Dose-dependent cell recruitment by citral. Data points were fitted with Hill Equation (RC$_{50}$ = 34.6μM, Hill coefficient = 0.71; N = 44).](image)

To determine the effective concentration range of Hedione, OSNs were stimulated with 30μM CIT with or without 10/30/100/300μM HED. 66 OSNs that were activated by 30μM CIT but not by 300μM HED alone were utilized for further analysis. The modulation indices ($I_{mod}$) were calculated at each concentration and shown as a heatmap (Figure 13). The number (percentage) of OSNs showing enhancement ($I_{mod} > 0.3$) was 4(6%), 4(6%), 3(6%) and 3(6%) when Hedione concentration was at 10/30/100/300μM, respectively; the number (percentage) of OSNs showing inhibition ($I_{mod} < -0.3$) was 7(11%), 9(14%), 21(32%) and 41(62%). While
enhancement was rare across all concentrations, inhibition was frequently observed and became more widespread as HED concentration increased. An example of HED inhibiting neuronal response to CIT is shown in Figure 14.

**Figure 13. Dose-dependent modulation effects of Hedione.** OSNs were stimulated with 30μM CIT, with or without 10/30/100/300μM of HED. Modulation indices ($I_{mod}$) were calculated at each concentration. (N = 66)

**Figure 14. Time course of an individual OSN showing dose-dependent inhibition by Hedione.**
The dose dependency of inhibition suggested a competitive mechanism. I then asked if OSNs with different CIT affinities were modulated differentially by HED. In this set of experiments, OSNs were stimulated with 1/10/100/1000\(\mu\)M of CIT with or without 100\(\mu\)M HED. 129 OSNs that were activated by 100\(\mu\)M CIT but not by 100\(\mu\)M HED were used for analyses. The modulation indices \((I_{mod})\) were calculated for each OSN at 1/10/100\(\mu\)M of CIT (Figure 15A). Examples of individual neurons showing different CIT affinities can be seen in Figure 15B. To evaluate the relationship between the modulation effect and CIT affinity, each neuron’s \(I_{mod}\) at 100\(\mu\)M CIT was plotted against its corresponding EC\(_{50}\) value (Figure 15C). As EC\(_{50}\) increased, more OSNs were inhibited, indicating that HED functioned as a weak antagonist.

The same experiment was then repeated with 3/10/30/100\(\mu\)M of EUG and 100\(\mu\)M HED. Compared with CIT-activated OSNs, EUG-activated OSNs seem to be more enhanced and less inhibited (Figure 16A-B). Nevertheless, OSNs with higher Eugenol EC\(_{50}\)s were more likely to be inhibited than those with lower EC\(_{50}\)s (Figure 16C), consistent with previous results from CIT.
Figure 15. Hedione effects on OSNs with various citral affinities. (A) Heatmap of the Hedione (HED) modulation effects (N = 129). OSNs were stimulated with 1/10/100/1000μM of citral (CIT), with or without 100μM of HED. Each row represents a single neuron and each column represents the effect of HED at corresponding CIT concentrations. (B) Time courses of neurons showing high/medium/low affinities to CIT. (C) Correlation between CIT EC$_{50}$ and HED modulation effects. Each datapoint represents the modulation index at 100μM CIT of one single OSN. Result of linear fitting: $y = -0.05x - 0.11$, $R^2 = 0.015$. 
Figure 16. Hedione effects on OSNs with various eugenol affinities. (A) Heatmap of the Hedione (HED) modulation effects (N = 80). OSNs were stimulated with 3/10/30/100μM of eugenol (EUG) with or without 100μM of HED. Each row represents a single neuron and each column represents the effect of HED at corresponding EUG concentrations. (B) Example time courses of three different neurons. (C) Relationship between EUG EC₅₀ and HED modulation effects. Each datapoint represents the modulation index at 100μM EUG of one single OSN.
3.3.3. **Characterizing molecular features critical for the Hedione modulation effects**

The next question is to identify the key features that make Hedione a weak antagonist. Three sets of experiments were designed to answer this question: first, compare Hedione with its stereoisomeric components; second, compare Hedione with dehydro-hedione, a conformation-restricted analogue of Hedione; finally, compare Hedione with two partial-analogues, methyl nonanoate and cyclopentanone.

The first set of experiments aimed to evaluate the importance of chiral centers. The molecular structure of Hedione contains two chiral centers in position 1 and 2 of the cyclopentanone ring, resulting in four possible stereoisomers. Since the pure forms are not readily accessible, three mixtures of these stereoisomers at different ratio were utilized for comparison: All-trans Hedione (an equalmolar mixture of the two *trans* diastereomers), Paradisone (predominantly the *(1R, 2S)*-Hedione) and Hedione (mix of all four stereoisomers; see Figure 17).

**Figure 17. Structures of Hedione stereoisomers**. The chiral center formed by the ester arm is designated as 1 and the chiral center formed by the alkyl arm is designated as 2. Hedione is consisted of 90% of the *trans* diastereomers (45% each) and 10% of the *cis* diastereomers (5% each); All-trans hedione contains only the two *trans* diastereomers at 1:1 ratio; Paradisone almost exclusively contains the *(1R,2S)*-Hedione.
To compare the effects of the stereoisomers, OSNs were stimulated with 30μM of CIT alone or mixed with 100μM of All-trans Hedione (TRANS), Paradisone (PARA) or Hedione (HED). Example time course of an individual OSN is shown in Figure 18. Responses to the isomers alone and the modulation effects of each isomer were calculated and summarized in Figure 19. In the activation panel, cell responses to TRANS and HED were more correlated than responses to PARA (Table 4). In the modulation panel, all three compounds could function as both inhibitor and enhancers. Again, the modulation effect of PARA was less correlated with TRANS and HED (Table 5). The higher similarity between TRANS and HED was probably due to their higher similarity in the ratio of isomers. Meanwhile, the different response profile of PARA indicated that the agonistic and the modulation spectrums was shifted by changes in chirality.

Figure 18. Time course of an individual OSN showing the modulation effects of Hedione stereoisomers. Cells were first stimulated with 100μM of All-trans hedione (TRANS), Paradisone (PARA) or Hedione (HED) individually as agonists (Activation Panel). Cells were then stimulated with 30μM CIT and its mixtures with TRANS/PARA/HED, respectively (Modulation Panel). The OSN shown here was completely inhibited by TRANS and HED, but only weakly suppressed by PARA.
Figure 19. The agonistic and modulation effects of Hedione and its stereoisomers. Each row represents an individual neuron. Columns 1-3 (Activation Panel) compare the agonistic effects of All-trans hedione (TRANS), Paradisone (PARA) and Hedione (HED); color intensity indicates response magnitude (normalized to maximum odor response). Columns 4-6 (Modulation Panel) compare their modulation effects on responses to 30μM CIT; colors represent the corresponding modulation indices ($I_{mod}$). (N = 90)
Table 4. **Comparison of Hedione stereoisomers as agonists.** Correlation coefficients were calculated based on OSN responses to TRANS, PARA and HED (denoted as “activation panel” in Figure 19).

<table>
<thead>
<tr>
<th></th>
<th>TRANS</th>
<th>PARA</th>
<th>HED</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRANS</td>
<td>1</td>
<td>-0.07</td>
<td>0.69</td>
</tr>
<tr>
<td>PARA</td>
<td>-0.07</td>
<td>1</td>
<td>-0.01</td>
</tr>
<tr>
<td>HED</td>
<td>0.69</td>
<td>-0.01</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5. **Comparison of Hedione stereoisomers as modulators.** Correlation coefficients were calculated based on the modulation indices ($I_{mod}$) of TRANS, PARA and HED (denoted as “modulation panel” in Figure 19).

<table>
<thead>
<tr>
<th></th>
<th>TRANS</th>
<th>PARA</th>
<th>HED</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRANS</td>
<td>1</td>
<td>0.92</td>
<td>0.91</td>
</tr>
<tr>
<td>PARA</td>
<td>0.92</td>
<td>1</td>
<td>0.84</td>
</tr>
<tr>
<td>HED</td>
<td>0.91</td>
<td>0.84</td>
<td>1</td>
</tr>
</tbody>
</table>

The second set of experiments compared HED with dehydro-hedione (DHH), in which the two chiral centers were flattened by linking them with a double bond. Among the 60 OSNs that were odor-responsive, both the activation profile and modulation profile of DHH were highly consistent with those of HED (Figure 20). This result, in combination with the result from the previous dataset, suggested that moderate changes in the conformation of HED are generally tolerated by the receptors, while certain conformation (such as that of PARA) may lead to greater functional changes.
Figure 20. The agonistic and modulatory effects of Hedione and dehydro-hedione. Each row represents an individual neuron. Columns 1-2 compare the agonistic effects of dehydro-hedione (DHH) and Hedione (HED); color intensity indicates response magnitude (normalized to the maximum odor response). Columns 3-4 compare their modulatory effects on 30μM CIT; colors represent the corresponding modulation indices ($I_{mod}$). (N = 60)
The third set of experiments compared HED with methyl nonanoate (MN) and cyclopentanone (CYP). These two molecules are essentially “fragments” of HED: MN mimics the two arms of HED, while CYP mimics the ring (Figure 21). As in previous experiments, HED and its analogues were presented to OSNs individually as agonists, then mixed with 30μM CIT to evaluate their modulation effects (Figure 22).

![Figure 21. Structures of Hedione and its fragmental analogues.](image)

Figure 22. Time courses of two OSNs showing the effects of Hedione and its fragmental analogues. Cells were first stimulated with 100μM of Cyclopentanone (CYP), Methyl nonanoate (MN) or Hedione (HED) individually as agonists (Activation Panel), then stimulated with 30μM CIT or its mixtures with CYP/MN/HED, respectively (Modulation Panel). One OSN (top) showed strong activation by MN as an agonist, the other (bottom) showed partial suppression by MN and complete inhibition by HED, respectively.
34 odor-responsive OSNs were yielded for analyses. 22 neurons were directly activated by HED and/or its analogues; specifically, 11 were activated by HED, 3 by CYP and 21 by MN (Figure 23, activation panel). Notably, all HED-activated OSNs were also activated by MN. This was probably because of the much higher conformational flexibility of MN, while the MN-like backbone is restricted by the cyclopentanone ring in HED. The strong activating effect of MN is also consistent with its strong, plastic-like smell by human perception. Among the same 34 OSNs, 8 were inhibited by HED, 0 by CYP and 2 by MN (Figure 23, modulation panel). While CYP did not show any inhibitory effect, MN seemed to weakly suppress neuronal responses to 30μM CIT (Figure 22 bottom and Figure 23). Taken together, the current dataset suggested that the two arms in HED participated in both activation and modulation of odor responses, while the cyclopentanone ring was relatively inert and prevented HED from being a strong agonist.

Figure 23. The agonistic and modulatory effects of Hedione and its fragmental analogues. Each row represents a single neuron. Columns 1-3 (Activation Panel) compare the agonistic effects of Cyclopentanone (CYP), Methyl nonanoate (MN) and Hedione (HED); color intensity indicates response magnitude (normalized to maximum odor response). Columns 4-6 (Modulation Panel) compare their modulatory effects on 30μM CIT; colors represent the corresponding modulation indices ($I_{mod}$). (N = 34)
HED is not the only compound known to have modulation effects on other odors. Fructalate (diethyl cyclohexane-1,4-dicarboxylate, abbreviated as FRUC) is also a frequently used additive in perfumery. It imparts a raspberry smell by itself, but also functions as “booster” in odor blends, especially in fruity accords. Interestingly, the molecular structures of FRUC and HED have some features in common: they both have a scaffold consists of one ring and two arms, at least one arm bearing an ester group (Figure 24). The functional and structural similarity raises the possibility that FRUC modulates odor responses in a way similar to HED.

![Figure 24. Chemical structures of Hedione and Fructalate.](image)

To test this hypothesis, I evaluated the modulation effects of HED and FRUC on an odor blend containing citral (CIT), γ-terpene (gTER) and isoamyl acetate (IAA), each at 30μM. The reason for using a mixture instead of an individual odor was to expand the receptor activation range. The sequence of odor stimuli and example time courses were shown in Figure 25.
Figure 25. Time courses of individual OSNs showing the effects of Hedione and Fructalate. OSNs were first presented to 100μM of Fructalate (FRUC) and Hedione (HED), respectively (the “Activation Panel (1)”). The modulation effects of FRUC and HED were then tested with a mixture containing CIT (CIT), γ-terpene (gTER) and isoamyl acetate (IAA), each at 30μM (the “Modulation Panel”). To identify the activating component in the mixture, cells were then stimulated with 30μM of CIT, gTER and IAA individually (the “Activation Panel (2)”), followed by 50μM forskolin (FORK) to confirm cell viability.

A total of 70 OSNs were odor responsive and utilized for further analyses. Among these neurons, 9 were activated by FRUC and 11 by HED; only 2 OSNs responded to both chemicals (Figure 26, activation panel). The lack of overlap between FRUC- and HED-activated OSN population might explain the distinct odor qualities of these two compounds (raspberry vs jasmine). Despite of the disparity in their activation profile, both FRUC and HED were able to inhibit OSN responses to the three-odor mixture (Figure 25, top; Figure 26, bottom rows); in addition, their modulation effects seemed to be correlated (Figure 26, modulation panel), though not always consistent (Figure 25, bottom). This similarity between FRUC- and HED-elicited modulation effects is intriguing, especially given their barely overlapping spectrum as agonists.
Figure 26. The agonistic and modulatory effects of Hedione and Fructalate. Each row represents a single neuron. Columns 1-6 (Activation Panel) compare cell responses to Fructalate (FRUC), Hedione (HED), Citral (CIT), γ-terpene (gTER), isoamyl acetate (IAA) and the CIT-gTER-IAA mixture (MIX); color intensity indicates response magnitude (normalized to maximum odor response). Columns 7-8 compare the modulation effects of FRUC and HED on cell responses to MIX (Modulation Panel); colors represent the corresponding modulation indices ($I_{mod}$). (N = 70)
3.4. Discussion

In this project, I investigated the modulation effects of Hedione in the peripheral olfactory system. Data suggested that Hedione was capable of both enhancing and inhibiting neuronal responses to other odors, with inhibition being the dominating modulation effect. Moreover, Hedione seemed to function as a weak antagonist, which preferably suppresses receptors with lower affinities to the agonist odor. In pharmacology, weak antagonists are generally not considered useful drug candidates due to their lack of potency and specificity. Here, we find that Hedione could enhance the perception of other odors by acting as a weak antagonist, enhancing the contrast between “on” and “off” targets. This phenomenon inspires a new strategy of drug development. For example, drug candidate X is a potent agonist of targeted receptor A as well as a weak agonist of non-targeted receptor B and C. The non-specific activation of receptor B and C by X causes side effects. The conventional solution would be looking for new substitutes of X with higher specificity. However, leveraging the concept of weak antagonism, this problem could potentially be solved by adding another compound Y that is sufficient to antagonize X binding to its low affinity receptors B and C but not the high affinity receptor A. This alternative strategy could remove lots of limitations in chemical structures, making drug design more flexible.

The other finding is that Hedione is not the only modulator that acts through weak antagonism. In this study, not only Hedione’s stereoisomers and fragmental analogues, but also Fructalate was shown to modulate OSN responses. This suggested that a wide range of compounds may act as weak antagonists, especially the ones that share a similar scaffold with Hedione and Fructalate.

The experiments testing Hedione stereoisomers and dehydro-hedione were designed together with Dr. Zita Peterlin. As an ongoing project, data presented in this chapter are
preliminary and need to be repeated to generate a more comprehensive characterization of the Hedione.

One limitation of this study and studies in the previous chapters was the low throughput and temporal resolution of the imaging technique. This technique also requires the OSNs to be digested and coated on a coverslip before imaging, which inevitably causes damage to the neurons. These drawbacks motivated the development of a novel imaging method, as detailed in the following chapter.
Chapter 4

DEVELOPMENT OF SCAPE MICROSCOPY-BASED OSN IMAGING TECHNIQUE

4.1. Introduction to SCAPE microscopy

Ca$^{2+}$ is a ubiquitous second messenger that plays a central role in the signal transduction of various types of neurons, including OSNs. Recording changes in intracellular Ca$^{2+}$ signals has thus been a feasible way of monitoring OSN activity. Conventionally, calcium imaging of OSNs has been performed on a glass coverslip coated with a thin layer of dissociated OSNs (see Methods in Chapter 2). However, the acute OSN culture preparation has several disadvantages. First, the axons of OSNs are severed during dissection, and the remaining soma and cilia are also damaged during enzymatic digestion. This sample preparation method is also time consuming, typically requiring more than 6 hours. Second, the screening throughput and temporal resolution is relatively low. With the imaging system in our lab, ~100-200 OSNs can be visualized per field-of-view (FOV), depending on cell density. There is a trade-off between number of FOVs imaged simultaneously and acquisition speed: when imaging at 0.25Hz (one frame every 4s), the stage can move between 2-3 different FOVs, which means to image 200-600 OSNs simultaneously. Given that one odor
typically recruits ~5-10% of all OSNs, the number of OSNs activated by given odor stimuli is small, which makes it difficult to detect low-incidence events. Finally, once the OSNs are dissociated, they cannot be mapped back to the epithelium.

The disadvantages mentioned above have motivated seeking of a more effective and less harmful way of imaging OSNs. One seemingly apparent solution is to peel off a piece of epithelium and image it directly. However, a regular epi-fluorescent microscope can only focus on one single plane at a time. Although the epithelial tissue looks very thin at first glance, it still has a depth of ~150-200μm, across which OSNs are densely packed. Moreover, the epithelium surface is curvy, which makes it more difficult to focus. It is also difficult to stably mount the tissue, especially under perfusion.

The invention of Swept Confocally-Aligned Planar Excitation (SCAPE) microscopy has inspired a novel solution to this problem [121]. SCAPE is a lightsheet-based volumetric imaging technique, capable of acquiring large FOV (~0.5-1 mm) at high temporal resolution (over 10 volumes/s). SCAPE microscopy merges the lightsheet excitation and detection paths into a single stationery objective lens paradigm, which has greatly facilitated the mounting and imaging of non-transparent tissue (Figure 27). In collaboration with Prof. Elizabeth Hillman and her student Dr. Wenze Li, we have developed a SCAPE-based method to image intact mouse olfactory turbinates, visualizing 1,000s of OSNs at the same time.
Figure 27. Schematic of the SCAPE system. Blue lines represent the lightsheet excitation pathway, green lines represent the detection pathway. Abbreviations: PL, powell lens; CL, cylindrical lens; S, scan lens; T, tube lens; O, objective lens.

4.2. Experimental design and Methods

4.2.1. Animals and tissue preparation

Mice were housed and handled in accordance with protocols approved by Columbia University Institutional Animal Care and Use Committee. OMP-Cre-driven GCaMP6f strain was generated by crossing OMP-Cre strain (JAX006668) with Ai95D (CAG-GCaMP6f, JAX024105). For sample preparation, male OMP-Cre\textsuperscript{+/−} GCaMP6f\textsuperscript{−/−} mice at 6-8 weeks of age were overdosed with anesthetics (ketamine 90 mg·kg\textsuperscript{−1}; xylazine 10 mg·kg\textsuperscript{−1}, i.p.) and decapitated in accordance with IACUC approved procedures. The head was cut along the sagittal plane and the septum was removed to expose the surface of the olfactory turbinates. The right half of the hemi-head tissue
was placed in cold modified Ringer’s solution (mM: 113 NaCl, 25 NaHCO₃, 5 KCl, 2 CaCl₂, 3 MgCl₂, 20 HEPES, 20 Glucose, pH 7.4) for 40min before imaging.

In order to estimate the number of OSNs that could be imaged simultaneously with SCAPE, OMP-Cre⁺/⁻ GCaMP6f⁺/⁻ mice were crossed with Ai75D (RCL-nT, Jax025106) to generate an OMP-Cre⁺/⁻ GCaMP6f⁺/⁻ nuc-tdTomato⁺/⁻ strain, which co-expresses nuclear-localized tdTomato and GCaMP6f in mature OSNs. An 8-week old male mouse was imaged in dual-color mode (green and red) with SCAPE microscopy. 3D segmentation was applied to the acquired imaging volume to estimate the total number of OMP positive neurons.

### 4.2.2. Perfusion and odor delivery

A perfusion chamber was custom-designed and 3D-printed for better tissue mounting and flow control. Specifically, the bath area of this chamber was designed to mimic the outline of the mouse hemi-head, and the perfusion inlet was set close to the nostril and outlet to the throat (Figure 28, blue trace and arrows). After sealing the bottom of the chamber with a coverslip, the hemi-head tissue was mounted with the epithelial surface facing down to the glass bottom. Because of surface tension, the narrow space between the epithelium and the coverslip will be filled up by the perfused Ringer solution. This facing-down design allowed faster exchange of solution compared to facing-up. The tissue was then glued to the chamber with a small amount of light-cured dental composite (Tetric EvoFlow®, Ivoclar Vivadent) to minimize drifting during experiment. Finally, the chamber was sealed with a glass cover to prevent the non-immersed portion of tissue from drying out.

During experiments, the tissue was continuously perfused with carboxygenated (95% O₂, 5%CO₂) modified Ringer’s solution at room temperature using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA). The flow rate was set at 0.75ml·min⁻¹. Depending
on individual differences and the particulars of the tissue mounting, there was some variation in the precise region that was imaged. Typically, the field of view covered the ventral half of either turbinate IIb or III [38], and some portion of the neighboring turbinates (Figure 28, yellow rectangle).

Figure 28. Schematic of intact olfactory epithelium imaging platform for SCAPE microscopy. A custom-designed glass bottom perfusion chamber was placed above the inverted objective with water immersion. The right half of the mouse head was mounted in the perfusion chamber, with the olfactory turbinates exposed. The perfusion chamber was designed to control the perfusion flow through the nasal cavity with the inlet at the nostril and the outlet at the throat (blue arrows). The imaging area typically covered the ventral half of either turbinate IIb or III, and some of the neighboring turbinates (yellow rectangle).

Odors were applied for 30s using the 1260 Infinity HPLC system with a 2.5min time intervals between stimuli. The adenylate cyclase activator forskolin (50μM, Sigma-Aldrich) was applied at the end of each experiment to assess the viability of OSNs.

4.2.3. SCAPE imaging

High-speed volumetric imaging of the intact epithelium was performed on a custom Swept Confocally-Aligned Planar Excitation (SCAPE) microscope [122-124]. Briefly, the sample was illuminated with an oblique light sheet through a 1.0 NA primary objective lens. The emission
signal excited by this sheet (extending in y-z’) is collected by the same objective lens (in this case an Olympus XLUMPLFLN 20XW 1.0 NA water immersion objective with a 2mm working distance). A galvanometer mirror in the system is positioned to both cause the oblique light sheet to scan from side to side across the sample (in the x direction) but also to de-scan returning fluorescence light. This optical path results in an intermediate, de-scanned oblique image plane which is stationary yet always co-aligned with the plane in the sample that is being illuminated by the scanning light sheet. Image rotation optics and a fast sCMOS camera (Andor Zyla 4.2+) were then focused to capture these y-z’ images at over 800 frames per second as the sheet is repeatedly scanned across the sample in the x direction. All other system parts including the objective and sample stage are stationary during high speed 3D image acquisition. Data is reformed into a 3D volume by stacking successive y-z’ planes according to the scanning mirror’s x-position and de-skewing to correct for the oblique sheet angle.

In this study, the stationary objective in SCAPE was configured in an inverted arrangement to image underneath the perfusion chamber. The overall magnification of the system was configured to be 4.66x. A 488nm laser was used for excitation (<1.4 mW at the sample) with a 500 nm long pass filter in the emission path. The system’s sCMOS camera was used at various frame rate for different specific ROIs (800-1300 fps). The maximum field of view of the SCAPE system can be as large as 1600µm × 1200µm × 350µm (Figure 33). To achieve optimal spatial-temporal resolution, the sample was typically imaged with an x-direction scanning step of 2-3µm over a 1000µm × 600-800µm × 240µm field of view (y-x-z, 1.39×(2-3)×1.1µm per pixel) at 2-5 volumes per second. Due to the variation of FOV and the tissue structure, the number of cells acquired ranged from 6,000-12,000 per mouse. Each trial was acquired for 75 seconds.
4.2.4. Data processing

The data processing pipeline can be divided into the following steps:

Preprocessing

The raw SCAPe data are spool files and need a specific script to load into the workspace of Matlab (MathWorks, Boston, MA, USA). Since the specimen was scanned with an oblique light-sheet, each 3D volume needs to be de-skewed to the original scale. The raw volumes were loaded and de-skewed with custom Matlab scripts.

Image registration

Sample drifts during experiment were minimal and can be easily corrected with various imaging processing packages. However, the tissue also underwent deformation at the scale of μm, which needed to be registered non-rigidly. Due to the lack of apparent “landmarks” at this scale, registration with commonly used methods (such as Matlab build-in functions or FIJI packages) did not yield satisfactory results. To address this issue, I adopted the Non-Rigid Motion Correction (NoRMCorre) algorithm developed by Pnevmatikakis et al. [125] and custom-modified it to perform deformable registration specifically for our own dataset. The primary mechanism of NoRMCorre is to split a 3D volume into multiple overlapping sub-patches and calculate the rigid shift for each sub-patch. The piece-wise shifts are then up-sampled and smoothed to produce a final non-rigid displacement field for the entire image. Since tissue drift and motion artifact within each 75s trial was negligible, I took a single 3D volume (averaged from ten volumes during the resting state) from each trial and registered them to the template, which was also an averaged 3D volume from the reference trial. The displacement field was then applied to all the volumes in each trial. This modification has significantly reduced the calculation amount from the original frame-
by-frame update scheme. The default piece-wise registration method was replaced with a Matlab build-in function \textit{imregister} to improve the registration results for our dataset. Since the registration was based on gradient-descent and can sometimes result in ‘outlier’ output, I created a graphical use interface (GUI) to manually correct the registration result of each sub-patch when necessary (Figure 29).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{custom_user_interface.png}
\caption{Custom-designed user interface for supervised piece-wise registration. The 3D image pairs to be registered (700\,\mu m \times 120\,\mu m \times 430\,\mu m) were splitted into 437(19 \times 1 \times 23) sub-patches (36\,\mu m \times 120\,\mu m \times 18\,\mu m each). One individual sub-patch is displayed in x-y, x-z and y-z perspectives (columns left, middle and right) before and after registration (top and bottom rows). Each image is the overlay of the template (green) and the moving image (magenta). The panel on the right side controls rigid displacement at each dimension.}
\end{figure}

The overall registration results generated by the custom NoRMCorre scripts was overall satisfactory (Figure 30, middle). However, manually validating image sub-patches was labor-intensive. To overcome this bottleneck, I switched to the Advanced Normalization Tools (ANTs), an Insight Toolkit (ITK)-based open-source toolkit for image registration as well as segmentation,
template-building and many other functionalities. ANTs employs a strategy termed Symmetric image Normalization (SyN) for deformable image registration. In short, the SyN algorithm aligns two images by maximizing their cross-correlation with a symmetric diffeomorphic optimizer [126]. The application of diffeomorphism fits accurately with the situation of tissue deformation, while the cross-correlation-based loss evaluation also exhibits robustness in handling image pairs with dynamic changes. After the parameters were properly tuned, registration performance of this method was comparable with that of NormCorre, sometimes slightly better at resolving localized deformation (Figure 30). Although the SyN-based registration was still time-consuming (30-60 min per image pair), it did not require supervision and therefore can be automatically processed in batch through a custom bash script, which significantly accelerated the registration process.

Figure 30. Demonstration of two non-rigid registration methods. 3D volumetric images of the template (green) and the moving subject (magenta) were recorded from the same specimen with a 25min time interval. Both volumes were de-skewed and rigidly registered before non-rigid registration (top). The image pair were then registered with NormCorre (middle) or SyN (bottom) method. For visualization purpose, the figure only shows one frame of the whole volume (XY plane). Note that SyN works slightly better at resolving localized deformation.
Denoising, demixing and data sorting

OSNs are densely packed in the intact olfactory epithelium. Therefore, it is critical to eliminate the noise from neighboring cells when analyzing a given OSN. This was achieved through the application of Constrained Nonnegative Matrix Factorization (CNMF), a framework that implements denoising, deconvolution and demixing of calcium imaging data simultaneously [127]. CNMF is capable in processing both 2D and 3D data, yet the 3D version is computationally much more expensive and can only process a small volume (~200μm x150μm x50μm) each time, thus being less feasible than the 2D version. To prepare data suited for 2D CNMF, I divided the 3D volumetric data from each trial into multiple 7.7μm thick 3D sub-stacks with 3.3μm spacing to minimize overlapping of neurons. Each sub-stack was then projected into a 2D image series with the intensity summed through depth. This step would convert the 3D time series data into 10-20 2D image series depending on the shape of the imaged area. The 2D image series of the same depth section from different trials were then concatenated into a complete image series containing all the different odor stimuli and analyzed with CNMF. Forskolin trials were omitted from the initial CNMF analysis to extract only the neurons responding to the delivered odors and odor combinations. The same spatial components were then used to update the time course of each neuron to include the forskolin response. Since this algorithm inevitably also picks up numerous non-neuronal components and spontaneously activated neurons, the number of components for initialization is set to be higher than the expected number of responsive neurons, typically at 750-1500 per image series, among which 150-300 would be odor-responsive neurons.

To accelerate the data sorting process, I designed a 2D convolutional neural network to perform the initial screening of the CNMF-extracted time-courses and spatial loci. Specifically, the 1D time course of each CNMF-extracted component was reshaped to an $m \times n$ matrix (where
\( m \) is the number of trials and \( n \) is the number of data points in each trial) and down-sampled to an \( m \times 16 \) matrix (Figure 31). Using the 2D matrix as input, the convolutional neural network was able to capture the characteristic kinetics of genuine odor responses and classify the components as either ‘good’ (acceptable) or ‘bad’ (rejected) components with \( \approx 90\% \) of accuracy. During the automated screening process, components with spontaneous activity, motion induced baseline fluctuation or inconsistent responses to repetitive odor stimuli were excluded. The screening results were then manually validated with a custom-designed GUI to ensure data fidelity (Figure 32).

**Figure 31. Schematic of preparing inputs for the 2D convolutional neural network.** The normalized time course (1430 data points, left) of each component was reshaped to an \( 11 \times 130 \) matrix (middle) and then downsampled to an \( 11 \times 16 \) matrix (right). Note that a genuine odor responses (component #1) has a characteristic pattern while random noises (component #2) does not.
Figure 32. Custom-designed GUI for component validation. Each sub-plot displays the time course of one individual component. Buttons with red/blue colors indicate rejected/acceptable components classified by the supervised 2D convolutional neural network. Each button can be toggled between red/blue to manually validate the classification result of the corresponding component.

4.3. Proof-of-concept results

The hemi-head sample was prepared from an OMP+/--;GCaMP6f/- mouse strain that expresses Ca\textsuperscript{2+} indicator GCaMP6f specifically in mature OSNs. This preparation enabled a variety of odor stimuli to be perfused over the intact tissue while monitoring the simultaneous responses of thousands of olfactory neurons layered within the epithelium of the curved turbinates using SCAPE microscopy. The 3D volumetric rendering of high-resolution scanning and time courses of neural responses are shown in Figure 33 and Figure 34, respectively. A 3D rendering video showing dynamic neural responses to an odor stimulus (mixture of acetophenone, benzyl acetate and citral, 100μM each) has been supplemented as Appendix A2.
Since mature OSNs express only one allele of an odorant receptor gene [19], the response of a single neuron to a sequence of presented odor blends represents the specific properties of a particular receptor. Thus, our method permits characterization of the properties of a large population of receptors without requiring knowledge of the genetic identity of each individual receptor.

Figure 33. High-resolution scanning of the intact olfactory epithelium using SCAPE microscopy. A single volume was acquired from the olfactory epithelium at the resting level without odor stimulus showing a 1600µm×1200µm×350µm field of view. A zoom-in side view (Y-Z, orange box) and a top-down view (X-Y, yellow box) are shown on the right. Both views are the maximum intensity projections of 10µm sub-stacks. Scale bar, 50µm.
Figure 34. Odor-evoked GCaMP6f activities recorded using SCAPE microscopy. OSNs were stimulated with 100μM of acetophenone (Ace), Benzyl acetate (Ben) and Citral (Cit) subsequently and imaged at 5VPS. (A) Side view (Y-Z) of a 170μm x 460μm subregion showing neural activities. The image was calculated as the standard deviation of the three trials. (B) Time courses of three individual neurons. Cell #1-3 correlate to neurons circled in magenta/yellow/blue boxes in (A), respectively.

4.4. Discussion

This study was conducted in collaboration with Dr. Wenze Li, who provided support in SCAPE microscopy, hardware configuration of computers and data pre-processing. In this study, we utilized SCAPE microscopy to perform high throughput, high-speed 3D imaging of intact mouse olfactory epithelium. This approach allows us to image ~10,000 OSNs in parallel, far exceeding the possible read-outs with previous imaging methods or electrophysiological recordings. While the current experimental setup has been optimized for studying the encoding of peripheral olfactory information (as detailed in the next chapter), the SCAPE microscopy can be configured differently for other research purposes. The possibilities of alternative experimental designs and their advantages/disadvantages are discussed below.
4.4.1. Odor delivery options

There are two possible ways to deliver odors in vitro: vapor-phase and liquid-phase. In conventional calcium imaging experiments, dissociated OSNs need to be constantly perfused with Ringer’s solution to maintain their physiological functionality, while odors are also delivered through perfusion. In electroolfactogram (EOG) experiments, sample preparation is similar to that in SCAPE imaging, which also involves a hemi-head prep with turbinates exposed. Instead of perfusion, the EOG sample is streamed with humidified air and stimulated with odor puffs.

Both methods have advantages and disadvantages. Odor puffs have faster kinetics and resemble sniffing behavior more than liquid-phase delivery. However, vapor-phase odor is usually puffed out from the headspace of an odor solution container, in which case the concentration cannot be precisely controlled, especially when the components of an odor mixture have different saturated vapor pressures. In addition, a minimal waiting time must be allowed for the vapor-liquid system to reach equilibrium between stimuli. On the other hand, liquid-phase odor stimulus gains more precise concentration control at the price of slower kinetics. Liquid-phase odor delivery also has better compatibility with the water-immersion objective of the current SCAPE imaging system. Compared with air objectives, water-immersion objectives offer higher numerical apertures (NA) with better image resolution.

4.4.2. Selection of imaging area

The current hemi-head prep allows us to access olfactory turbinates I, IIa, IIb, and III. The septal wall and dorsal recess epithelium cannot be imaged with the same prep, but can be peeled off and glued on a coverslip, though being less healthy due to axonal injury. Alternatively, the dorsal recess can be accessed through a patch of thinned nasal bone from the top. Though not essential for our
current study, being able to image the dorsal regions is critical for studies that require a comprehensive profile of the dorsal OR repertoire, or those specifically targeting Class I ORs, in a living animal.

### 4.4.3. Glomerular imaging

OSNs expressing the same receptor send their axons to the olfactory bulb, forming one or more glomeruli [29-31], providing an alternative approach of investigating receptor activity. This is usually achieved by imaging the glomeruli through a cranial window. One advantage is that the experiments are performed *in vivo*. Besides, the signal-to-noise ratio at glomeruli is generally better than that at OSN soma. However, glomerular imaging usually focuses on the dorsal-most surface of the OB, which is ideal for studying Class I ORs [128], while ORs on the medial and ventral side of the OB are more difficult to access.

In summary, the SCAPE microscopy is a robust and versatile technique that can be adapted to meet a variety of research purposes. Since there is not a one-size-fits-all solution, the experiment design may vary case by case.
5.1. Introduction

The olfactory world is mostly composed of odor blends. However, during the past decades, most cellular olfactory investigations have used monomolecular stimuli to investigate the olfactory code. In this collaborative study with Prof. Elizabeth Hillman and her student Dr. Wenze Li, we performed high throughput analysis of single-cell responses to odor blends using SCAPE microscopy of intact mouse olfactory epithelium, imaging $\sim$10,000 olfactory sensory neurons in parallel. By stimulating OSNs with eight chemically distinct odors and their mixtures of up to three components, we discovered a variety of receptor-driven modulation effects, including antagonism, partial agonism and enhancement. As a result, the representation of the odor mix was distorted compared to a simple combinatorial sum of responses to each individual odor. Our results suggested a richer repertoire of receptor modulation mechanisms than previously thought.
5.2. Methods

5.2.1. Sample preparation and SCAPE imaging

Tissue samples were prepared and imaged as described in the previous chapter. Briefly, OMP-Cre\textsuperscript{+/−} GCaMP6f\textsuperscript{+/−} mice were euthanized in accordance with protocols approved by Columbia University Institutional Animal Care and Use Committee. Mouse head was dissected sagitally to expose the olfactory turbinates in cold modified Ringer’s solution (mM: 113 NaCl, 25 NaHCO\textsubscript{3}, 5 KCl, 2 CaCl\textsubscript{2}, 3 MgCl\textsubscript{2}, 20 HEPES, 20 Glucose, pH 7.4). For SCAPE imaging, the right half of the mouse hemi-head was mounted in a custom-designed 3D printed glass bottomed perfusion chamber and glued with a small amount of light-cured dental composite (Tetric EvoFlow\textsuperscript{®}, Ivoclar Vivadent). During experiments, the tissue was continuously perfused with carboxygenated (95% O\textsubscript{2}, 5%CO\textsubscript{2}) modified Ringer’s solution at 0.75ml·min\textsuperscript{−1}. The mounted sample was then placed over an inverted objective in SCAPE and imaged at 2-5 volumes per second. Each trial was acquired for 75 seconds, and each mouse was imaged for more than 20 trials with 2.5 min of inter-trial interval.

5.2.2. Odor stimuli

All odors in this study were from Sigma-Aldrich except Benzyl acetate, Dorisyl, Dartanol, and Isoraldeine which were gifts from Firmenich SA. In odor set 1, Acetophenone, Benzyl acetate and Citral were first diluted in DMSO to make stock solutions, then subsequently diluted in modified Ringer’s solution to 100μM. For two and three-component mixtures, Acetophenone, Benzyl acetate and Citral were mixed, then subsequently diluted so that each component had a final concentration of 100μM. In odor set 2, Dorisyl, Dartanol and Isoraldeine were mixed at a volume
ratio of 45% : 15% : 40% to reproduce the Woody Accord blend, an accord that has been widely used in the perfume industry. Final concentrations of the three odors were 148μM, 48μM and 127μM, respectively, both in single odor solutions and in mixtures. All odor solutions had a final DMSO concentration of 1-2.5‰ depending on the solubility of the odors; the DMSO concentration is balanced across all odor stimuli within the same experiment.

Odors were applied for 30s using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) with 2.5min time intervals between stimuli. For the 3-odor mix experiments, the sequence of individual and binary combinations of odors between the 3-odor mix stimuli was randomized among mice, although the 3-odor mix was always delivered 2nd, 6th and 10th to ensure repeatability and for correction of run-down when calculating the modulation index. Odor delivery in dose response experiments was performed in the fixed order shown in Fig. 4 and S5. The adenylate cyclase activator forskolin (50μM, Sigma-Aldrich) was applied at the end of each experiment to assess the viability of OSNs.

5.2.3. Volumetric image data processing

Sample drifts were corrected using custom Matlab code based on NoRMCorre [125]. Since tissue drift and motion artifact within each 75s trial was negligible, a single volume from each trial was taken and registered to a template with manual correction when necessary. The same transform matrix was then applied to all the volumes in each trial. After registration, 3D volumetric data from each trial were divided into multiple 7.7μm thick 3D sub-stacks with 3.3μm spacing to minimize overlapping of neurons. Each sub-stack was then projected into a 2D image series with the intensity summed through the 7.7μm depth of the sub-stack. The 2D image series of the same depth section from different trials were then concatenated into a complete image series containing
all the different stimuli, including DMSO and forskolin. After the pre-processing, the 3D time
series data were converted into 10-20 2D image series depending on the shape of the imaged tissue.
Constrained Nonnegative Matrix Factorization (CNMF) was then applied to each 2D image series
to extract single cell calcium activity [127]. Forskolin trials were omitted from the initial CNMF
analysis to extract only the neurons responding to the delivered odors and odor combinations. The
same spatial components were then used to update the time course of each neuron to include the
forskolin response.

CNMF-extracted time-courses and spatial loci were initially screened with a convolutional
neural network to exclude components with spontaneous activity, motion induced baseline
fluctuation or inconsistent responses to repetitive odor stimuli. All the cells were later validated
manually to ensure data fidelity.

Time courses of each neuron’s GCaMP activity are shown as $\frac{\Delta F}{F}$, where $\Delta F$ is the real-
time fluorescent intensity change and $F$ is the baseline fluorescent signal. Peak response
amplitudes were calculated as the average of a 3s window around the maximal value during odor
delivery, minus the median of a 6s window baseline extracted 15s before each odor was delivered.
Cells were considered to be exhibiting responses if their peak response amplitude exceeded 5x
standard deviations above the baseline noise.

5.2.4. Clustering cells and plotting normalized response heatmaps

Peak responses were calculated as detailed above and normalized for each cell to its maximum
odor response across all odors. Since odors were administered in a pseudo-random manner, data
from different animals were re-ordered before combination into the heatmap.
Neurons were clustered into subgroups using the k-means clustering function in Matlab with squared Euclidean distance. The number of clusters was determined for each dataset based on inspection and similar clusters were combined. For heatmap visualization, in some cases cells within each sub-group were re-ordered based on the primary odor response amplitude or their modulation index.

A more stringent criterion was applied when defining subgroups II-IV in Figure 37 and I-III in Figure 50 – that non-dominant odor responses must be 30% or less than the dominant odor response as detailed in the legend of Figure 37. Individual neuron responses within each of these clusters were visually inspected to ensure correct classification.

### 5.2.5. Calculating the modulation index

As shown in Figure 40, when calculating the modulation index \( I_{\text{mod}} \), responses to the mixture were linearly corrected based on responses to the two closest neighboring 3-odor mix responses. The \( I_{\text{mod}} \) for suppression effect was calculated as \( (d_0-d_1)/d_1 \), where \( d_0 \) was the linearly corrected response to the mixture and \( d_1 \) was the response to the dominant odor.

When calculating the enhancement effect of 3-odor mixture, this equation is modified to \( I_{\text{mod}}=\max( ((d_0-d_2-d_3)- d_1)/d_1, 0 ) \), where \( d_0 \) was the linearly corrected response to the mixture, \( d_1 \) was the response to the dominant odor and \( d_2, d_3 \) are the responses to the non-dominant odors (Figure 40). This different equation is intended to account for the amplitude of neuron responses to non-dominant odors, effectively removing those responses from the 3-mix response. This calculation may result in a lower enhancement modulation index by estimating a lower magnitude \( (d_0-d_2-d_3) \) than the actual 3-mix response without modulation, which might thus under-estimate the degree of enhancement while over-estimating the number of neurons showing no modulation.
effect. When calculating the enhancement effect for binary odor pairs (e.g. acetophenone paired with citral or other odors), the equation is modified to $I_{mod} = \text{max}( ((d_0 - d_2) - d_1)/d_1, 0 )$, where $d_0$ is the magnitude of the response to the binary pair, $d_1$ is the linearly corrected magnitude of the response to acetophenone alone and $d_2$ is the response to the modulator alone.

In the intact epithelial preparation used here, run-down of cell responses over time was comparable or less than that commonly experienced in dissociated cell preparations [120]. Figure 35 shows a control experiment for the linear run-down estimation with repeated delivery of the 3-odor mix. Here, a mock-modulation index ($I_{mod}$) was calculated as $(d_0 - d_1)/d_1$, where $d_0$ was the linearly corrected response to the 2nd mixture based on the neighboring mixture responses and $d_1$ was its real response to the mixture. In the ideal case, the calculated $I_{mod}$ would equal 0. Here, the mean of $I_{mod}$ was 0.002 with a standard deviation of 0.09 (N = 1302, 3 mice). A sample time course and the histogram of $I_{mod}$ is shown in Figure 35A and B, respectively.

Figure 35. Stable responses to repetitive odor stimuli. (A) Time course of an individual OSN. Neurons were stimulated repetitively by the ABC mixture (MIX, Acetophenone + Benzyl acetate + Citral, 100μM each) and DMSO. Cell viability was verified by 50μM forskolin (FORK). (B) Histogram showing variance of OSN responses. Modulation indices ($I_{mod}$) were calculated as $(d_0 - d_1)/d_1$, where $d_0$ was the linearly corrected response to the 2nd mixture based on the neighboring mixture responses and $d_1$ was its real response to the mixture. The results indicate that
although there are a few outliers, the actual responses of most OSNs were close to estimation, with a standard deviation of 9% (N = 1302, 3 mice).

5.3. Results

5.3.1. Response profile of odor set 1

The first odor set contained acetophenone (ACE), benzyl acetate (BEA) and citral (CIT), all at 100µM. These odor compounds were chosen so that they are distinct both chemically (Figure 36A) and perceptually (at least to humans). Specifically, the note of ACE is often described as almond or mimosa, BEA as floral or jasmine, and CIT as citrus. All three odors, especially ACE and CIT, are potent agonists that activate a large number of cells for analyses.

In pilot experiment, OSNs were stimulated with 100µM of ACE, BEA and CIT individually and as a mixture (MIX). Single cell analysis showed that, for most neurons, the magnitude of response to the mixture of all three odors was either same or slightly higher than the highest single odor response, which was expected for an additive code. One such cell is shown in Figure 36B (yellow box) and C. However, some neurons exhibited more complex response patterns. For example, the cell highlighted in Figure 36B (orange box) and C’ showed much weaker responses to the mixture than to ACE alone. This phenomenon suggested that while BEA and CIT did not (strongly) activate this cell, they might function as antagonists and inhibited cell responses to the mixture.
Figure 36. GCaMP time courses of individual OSNs extracted from raw SCAPE image. (A) Chemical structures of the three odors used as odor stimuli (odor set 1). (B) OSN responses to acetophenone (ACE, 100µM) alone and to the three-odor mixture (MIX, each at 100µM). Each image was cropped from a 1000µm×500µm×200µm volume, taken at peak responses. Two OSNs were highlighted: Scale bar, 20μm. (C, C’) The time courses of the highlighted OSNs (yellow and orange boxes in B). Data were extracted directly from the raw volumetric time series and calculated as \( \Delta F/F \). A 30s-long odor stimulus (MIX or each odor alone) was delivered in each trial, with a 2.5min interval between stimulus applications.

To assess the prevalence of this effect, we then performed a large-scale analysis on five different mice. In addition to the individual odor and three-component mixture stimuli, we also stimulated OSNs with the three possible binary pairs of odors to obtain a more comprehensive response profile. A total of 11,936 odor-responsive OSNs were yielded for analyses. Peak responses were calculated for each odor, and all responses for a given cell were normalized to the cell’s maximal response across all odors. The summarized data is plotted as a heatmap in Figure 37. Each row represents the response of a single neuron and each column represents an odor.
stimulus. Neurons were sorted into subgroups based on their response patterns using k-means clustering. This sorting process resulted in eight major subgroups based on their response patterns to the three individual odors. From top to bottom, these eight subgroups correspond to: cells activated by the three-odor mix, but only minimally activated by an individual odor (I), cells dominantly activated by one of the three individual odors (II-IV), cells dominantly activated by a binary pair of two of the three individual odors (V-VII), and cells activated by all three odors (VIII). Example time courses of individual neurons in each subgroup are shown in Figure 38.

Figure 37. Heatmap of normalized peak responses odor set 1. OSNs were stimulated by 100µM of ACE/BEA/CIT or their binary/ternary mixtures. Odor stimuli (columns) were given in a pseudo-random manner for each mouse and re-aligned for this presentation as denoted by the colored squares at the top. OSNs (rows) were clustered into eight subgroups (I-VIII) based on k-means clustering (N = 11,936, 5 mice).
Figure 38. Time courses of OSNs from subgroup I-VIII in response to odor set 1. An OSN was selected from each subgroup to illustrate different response patterns. Responses to 100μM acetophenone (ACE)/benzyl acetate (BEA)/citral (CIT) were highlighted with pink/blue/yellow rectangles, respectively.

Among the subgroups, cells dominantly activated by one of the three individual odors (Figure 37, subgroups II-IV) permit straightforward comparison between responses to that individual odor and responses to the mixture containing that odor. The dominate odor responses of most OSNs were unaffected by the other two components in the mixture (example time course in Figure 39, cell iii). However, numerous exceptions have been observed. For example, some cells in subgroup II (the ACE-dominant group) showed inhibited or suppressed responses to the
mixture (cell i and ii). Conversely, responses of some cells were enhanced. For example, in cell iv, BEA strongly enhances the response to ACE; cell v does not respond to any of the three odors individually, but can be activated by the binary mixture of ACE and BEA. Similar suppression and enhancement effects were also observed in BEA and CIT dominant subgroups (III and IV).

![Figure 39. Time courses of individual OSNs in subgroups I and II showing inhibition/suppression.](image)

Cell i/ii, responses to ACE inhibited/suppressed by CIT; iii, no modulation effect; iv, responses to ACE enhanced by BEA; v, “emergent” enhancement primarily by ACE and BEA.

To quantify these effects, a modulation index ($I_{mod}$) was calculated for each OSN to represent the relationship between response to the three-odor mixture and to its dominant single-odor response. An $I_{mod}$ value of -1 represents complete suppression, i.e., that a single-odor responding neuron has no response to the 3-odor mixture. An $I_{mod} >1$ represents more than two-fold enhancement of the response to the 3-odor mixture compared to the dominant single-odor response (Figure 40, see Methods for full details).
Figure 40. Quantification of the modulation effects. For suppression, a modulation index ($I_{mod}$) was calculated as $(d_0 - d_1)/d_1$, where $d_0$ was the corrected response to the mixture and $d_1$ was the dominant single odor response. When calculating enhancement, the formula was modified to $I_{mod} = \max\left( \frac{(d_0 - d_2 - d_3) - d_1}{d_1}, 0 \right)$, where $d_2$ and $d_3$ were responses to non-dominant odors. When $(d_0 - d_2 - d_3) - d_1/d_1$ resulted in a negative value, the $I_{mod}$ value is set to zero to avoid misinterpretation. Note that this method may underestimate the effect of enhancement.

The distributions of these modulation effects for each single-odor dominant subgroups (II-IV) are plotted in Figure 41. Most of the cells showed no modulation by the other odors in the mixture. However, for ACE, BEA and CIT-dominant cells, we found 19%, 3% and 3% of OSNs respectively showed $I_{mod} < -0.3$ (>30% suppression), and 20%, 23% and 27% of OSNs showed $I_{mod} > 0.3$ (>30% enhancement) respectively. Suppression effects were considerably stronger in ACE-dominant cells, whereas responses of BEA and CIT-dominant cells were more likely to be enhanced.

Figure 41. Representations of the modulation effects on ACE/BEA/CIT dominant neurons (subgroups II, III, IV). Red indicates that cell responses to the individual odors were inhibited by the mixture and blue indicates enhancement.
5.3.2. Dose response analysis of inhibition

To gain a deeper understanding of the mechanism underlying the modulation effects, we performed a series of dose-response experiments with ACE as an agonist and CIT as a modulator. A total of 11,774 OSNs across 5 mice were activated by either component in this binary odor pair (Figure 42). To explore suppression effects, we focused our analysis on a sub-population of 2,620 cells that showed little or no response to CIT alone. After k-means clustering and data sorting (see Methods), we found 410 OSNs (16%) that exhibited a suppressed ($I_{\text{mod}} < -0.3$) or completely inhibited response to ACE in the presence of 100μM CIT (Figure 43).

![Figure 42. Dose-dependent responses to acetophenone and citral.](image)

Figure 42. Dose-dependent responses to acetophenone and citral. Heatmap of normalized peak responses ($N = 11,774$, 5 mice) to acetophenone (ACE) and citral (CIT). OSNs were stimulated with increasing concentrations of acetophenone (10-300μM) in the presence or absence of 100μM citral. OSNs were sorted based on k-means clustering
results and the modulation index. Brackets of suppression and enhancement indicate neuron subgroups shown in Figure 43 and Figure 46, respectively.

**Figure 43. Dose-dependent suppression of acetophenone by 100μM citral.** (A) Normalized response heatmap of Acetophenone-activated neurons suppressed by Citral (N = 410). Neurons were stimulated by an increasing concentration of ACE (10-300μM) in the presence or absence of 100μM CIT as denoted by colored squares at the top. (B) Time course of an individual OSN showing suppression by 100μM CIT.

To generate dose-response curves, the averaged responses of all 410 OSNs with and without CIT are plotted against ACE concentration (Figure 44). It shows that the suppression effect of CIT could be outcompeted by increased concentrations of ACE, consistent with the mechanism of a competitive antagonist. In a few cases (28/410), CIT could both activate the neuron and suppress the neuron’s response to ACE (Figure 45). This response pattern fits the standard model of partial agonism.
Figure 44. The effect of 100μM citral as an antagonist. Dose-dependent responses (mean±S.E.M.) were plotted and fitted with the Hill Equation. Responses to ACE alone were plotted in black (Hill coefficient = 1.34, EC50 = 23.0μM), to ACE + CIT in red (Hill coefficient = 1.99, EC50 = 101.2μM).

Figure 45. Time course of an individual OSN showing partial agonism by 100μM citral.

5.3.3. Dose response analysis of enhancement

Supra-additive enhanced responses are more difficult to explain mechanistically than antagonism. An enhanced response to an agonist is generally ascribed to an allosteric mechanism [129]. However, allosteric modulation is rarely seen in classic small molecule Class A GPCRs. From the same subset of 2,620 cells described in the previous section (5.3.2), we identified 301 OSNs whose responses to ACE were enhanced by 100μM CIT (I_{mod} > 0.3; Figure 46A). In most cases, ACE
alone only activated OSNs at high concentrations, while ACE mixed with 100μM CIT could elicited OSN responses at lower concentrations (Figure 46B).

Figure 46. Dose-dependent enhancement of acetophenone by 100μM citral. (A) Normalized response heatmap of ACE-activated neurons enhanced by CIT (N = 301). (B) Time course of an individual OSN showing enhancement.

Among these neurons, 182 showed a small response to 100μM CIT alone, suggesting that the enhancement could either be a synergistic effect of mixing ACE and CIT together or the result of ACE enhancing CIT. The remaining 119 OSNs did not show a response to 100μM CIT and were utilized to plot the dose-response curves (Figure 47). This plot shows a clear shift to the left (enhancement) in the presence of CIT. Since these cells were not activated by CIT alone, the mechanism of enhancement is most likely an allosteric effect. In particular, some neurons were not activated by either CIT or ACE alone, even at the highest ACE concentration (300μM); however, the mixtures of 100μM CIT with low concentrations of ACE were sufficient to elicit activity (one such example is shown in Figure 48). This argues against the enhancement resulting just from increased ligand concentration at the orthosteric binding site. This effect may also explain the sub-set of cells seen in Figure 37, subgroup I. In this subgroup, cells had no or minimal responses to any of the individual odors (all at 100μM concentrations) but did generate a response
to the three-odor mixture, further indicating an allosteric function for one or more of the odors in the blend.

**Figure 47. The effect of 100μM citral as an enhancer.** Only the 119 OSNs with no baseline response to 100μM CIT were used to plot the dose-dependent curves (mean±S.E.M.). Responses to ACE alone were plotted in black and ACE + CIT in red, respectively. ACE alone: Hill coefficient = 1.95, EC₅₀ = 125.1μM; ACE + CIT: Hill coefficient = 0.87, EC₅₀ = 44.1μM.

**Figure 48. Time course of an individual OSN showing enhancement.** This OSN was not responsive to ACE alone at all concentrations and only showed noise-level response to 100 μM CIT, but was strongly activated by the mixtures of 100μM CIT and 10μM CIT or higher concentrations of ACE. This response pattern is possibly a result of positive allosteric modulation.
Although small molecule allosteric modulation of Class A GPCRs has been only rarely observed [130-135], the wide diversity of the OSN receptor family appears to have extended the occurrence of this mechanism in GPCRs of this type. We are unable from these data to determine the allosteric site. However, the odor molecules are relatively hydrophobic and from a chemical perspective, could easily access the lipid membrane and bind to sites within the transmembrane regions of the receptor or alter the lipid membrane environment.

5.3.4. Responses profile of odor set 2

In previous experiment with odor set 1 we found extensive and diverse modulation effects. We next asked if this is also the case with natural or composed blends. Therefore, we performed a similar analysis using a three-odor blend known as ‘Woody Accord’. It was designed by perfumers to impart a pleasant and harmonious woody scent to human perceivers. This specific formula contains 148μM dorisyl (DORI), 48μM dartanol (DART) and 127μM isoraldeine (ISO). The chemical structures of all three compounds are shown in Figure 49.

![Chemical structures of components in odor set 2.](image)

Figure 49. Chemical structures of components in odor set 2.
We first characterized neuronal responses to the three individual woody compounds and their binary/ternary mixtures. A total of 1,303 OSNs over 3 mice were odor-responsive (Figure 50A). OSNs were sorted and clustered into seven subgroups (I-VII). The response patterns of the seven subgroups of odor set 2 correspond to the subgroups II-VIII of odor set 1, while no match was found for subgroup I (the “emergent” enhancement group) of odor set 1 (Figure 37). A large proportion of DORI-dominant neurons were suppressed (Figure 50A, black box); comparison of OSN responses to DORI/DART (triangle) and DORI/ISO (asterisk) mixtures further indicated that ISO was the major inhibitor. Examples of single neuron responses are shown in Figure 50B.

Figure 50. Response profile of odor set 2. (A) Heatmap of normalized peak responses (N = 1303, 3 mice) to the Woody Accord. Odor stimuli (columns) were given in a pseudo-random manner and re-aligned for presentation clarity. The 7 subgroups were determined based on k-means clustering as for odor set 1. Suppressed OSNs in the DORI-
dominant group were highlighted (black box). Responses to DORI-DART (triangle) and DORI-ISO (asterisk) pairs were marked for comparison. (B) Sample time courses of individual OSNs.

Compared to response profile of odor set 1, odor set 2 showed more inhibition and less enhancement. Within the DORI, DART and ISO-dominant subgroups (I-III), we found 15% / 4% / 33% neurons showing suppression ($I_{\text{mod}} < -0.3$), and 9% / 5% / 3% neurons showing enhancement ($I_{\text{mod}} > 0.3$), respectively. The modulation indices for these subsets are calculated and summarized in Figure 51.

![Figure 51](image)

**Figure 51.** Histograms showing the distribution of modulation effects. DORI/ISO/DART-dominant neurons were analyzed and plotted in left, middle and right figures, respectively. Red indicates cell responses to individual odors were inhibited by the mixture and blue indicates enhancement.

We next performed the dose-response analyses with the DORI-ISO odor pair. OSNs were presented with 10-300μM of DORI with or without 100μM of ISO. A total of 3,396 of odor responsive neurons were yielded over 3 mice; a subset of 1,692 cells showed little or no response to ISO alone (Figure 52). Among these cells, 306 OSNs were inhibited by ISO ($I_{\text{mod}} < -0.3$, Figure 53); dose-response analysis indicated competitive antagonism (Figure 54). Additionally, 158 cells exhibited enhancement ($I_{\text{mod}} > 0.3$), 106 of which showed no baseline response to ISO alone and were utilized to plot dose-response curves. These enhancement effects are consistent with the
interactions between ACE and CIT described in previous section (Figure 55-56), reinforcing the possibility that allosteric modulations could occur between different odor pairs.

**Figure 52. Dose-dependent responses to dorisy1 and isoraldeine.** Neurons (N = 3396, 3 mice) were stimulated by an increasing concentration of DORI (10-300μM) in the presence or absence of 100μM ISO. 306 neurons were suppressed by more than 30%. 158 OSNs were enhanced by more than 30%.
Figure 53. **Dose-dependent suppression of dorisyl by 100μM isoraldeine.** (A) Normalized response heatmap of DORI-activated neurons suppressed by 100μM ISO (N = 306). (B) Time course of an individual OSN showing suppression.

Figure 54. **The effect of 100μM isoraldeine as an antagonist.** Dose-dependent responses (mean±S.E.M.) were plotted and fitted with the Hill Equation. Responses to DORI alone were plotted in black (Hill coefficient = 1.87, EC\textsubscript{50} = 105.2μM), to DORI + ISO in red (Hill coefficient = 1.24, EC\textsubscript{50} = 319.1μM).
Figure 55. Dose-dependent enhancement of dorisyl by 100μM isoraldeine. (A) Normalized response heatmap of DORI-activated neurons enhanced by 100μM ISO (N = 158). (B) Time course of an individual OSN showing enhancement.

Figure 56. The effect of 100μM isoraldeine as an antagonist. Only the 106 OSNs with no baseline response to 100μM Isoraldeine were used to plot the dose-dependent curves. Dose-dependent responses (mean±S.E.M.) were plotted and fitted with the Hill Equation. Responses to DORI alone were plotted in black (Hill coefficient = 1.73, EC50 = 42.4μM), to DORI + ISO in red (Hill coefficient = 1.80, EC50 = 6.9μM).
5.3.5. Additional modulators of ACE responses

How widespread is receptor modulation? While we have used a simple blend of a just a few odors and further concentrated on one or two of them as modulators, there is nothing inherently special about any of these molecules, which means response modulation could also occur between any pair of odors that happen to activate/antagonize/enhance the same receptor. If this is correct, CIT should not be the only antagonist of acetophenone (ACE). To test this hypothesis, we selected four different odors: dartanol (DART), isoraldeine (ISO), γ-terpinene (gTER) and isoamyl acetate (IAA) and paired them with ACE. The chemical structures of all five odor compounds used are shown in Figure 57.

![Chemical structures of acetophenone and odors tested as modulators.](image)

We identified 6,178 cells across 3 mice that were activated by at least one of these odors or their binary mixtures (Figure 58); among which, 1,309 were activated by Acetophenone. After k-means clustering and cell sorting based on different response patterns to different odor stimuli, 80 of these cells (6%) showed suppression ($I_{mod} < -0.3$) by one or more of the other compounds (Figure 59). As denoted by the colored boxes in the figure, cell responses to ACE were suppressed by one or more of the other compounds, ISO being the most common. Interestingly, these suppressed OSNs also showed diversified response patterns to the four modulators, indicating that they express multiple types of receptors. For example, cells i and ii in Figure 60 were both inhibited
by ISO, but showed differential responses to DORI. In rarer cases, cells were inhibited by more than one odor (cell iii), or even suppressed by all four odors equivalently (cell iv).

Figure 58. Heatmap of normalized peak responses to acetophenone and four modulators. OSNs were first stimulated with 100μM Dorisyl (DORI), Isoraldeine (ISO), γ-terpinene (GTER) and Isoamyl acetate (IAA) without Acetophenone (ACE) and then with 30μM ACE. Odor stimuli were presented in a pseudo-random manner and re-aligned. OSNs were sorted based on k-means clustering results and sorting of modulation index. Brackets of suppression and enhancement indicate neuron subgroups shown in Figure 59 and 60, respectively. (N = 6178, 3 mice)
Figure 59. Normalized response heatmap of suppressed OSNs. Odor stimuli are denoted by colored squares at the top (color coding is denoted on the top right corner). The columns of the heatmap were reordered for easier visualization of the suppression effects. Neurons showing suppression effects are boxed in different colors corresponding to the different modulators. (N = 80)

Figure 60. Time courses of four OSNs showing suppression. Responses to 30μM ACE alone are highlighted by pink rectangles; arrows with different colors indicate suppression/inhibition by the corresponding odors.
We also observed 73 cells showing enhancement ($I_{mod} > 0.3$) among the same 6,178 cells. Their response patterns were shown in Figure 61 with enhancement effects highlighted in boxes. Some example time courses of individual OSNs showing enhancement by different modulators can be seen in Figure 62. Cell vii is of particular interest in that it did not respond to ISO, gTER or ACE, but both binary odor pairs (ISO/ACE and gTER/ACE) did activate the neuron. Thus, it appears that a wide variety of molecules, both those with and without an apparent smell, could potentially act as modulators at different receptors.

**Figure 61. Normalized response heatmap of enhanced OSNs.** The columns of the heatmap were reordered for easier visualization of the enhancement effects. OSNs showing enhancement effects were boxed in different colors corresponding to the different modulators. ($N = 73$)
Figure 62. Time courses of four OSNs showing enhancement. Arrows with different colors indicate enhancement by the corresponding odors.

5.4. Discussion

In this study we have observed widespread modulation of peripheral sensory responses to odor mixtures, including both suppression and enhancement. Antagonism at the receptor level has been occasionally observed, and multiple mathematical and biophysical models have been proposed to describe the consequences of mixing odors [117-119, 136-142]. However, the low throughput of classical methods to assess cell-specific responses to multiple odors has made it difficult to perform comprehensive investigations of odor interactions in mixtures. Making use of a new high-throughput, high-speed 3D imaging technology (SCAPE microscopy), we demonstrated that the receptors themselves are engaged in a variety of modulatory responses, including antagonism, partial agonism and enhancement, before any further synaptic mediated processing of the stimulus.
at higher system levels. In addition, this study also revealed the occurrence of likely allosteric enhancement at classic small molecule Class A GPCRs, an effect long searched for but only rarely observed among this group of receptors [130-135].

Our results demonstrate that odors can act as agonists at one receptor and antagonists or partial agonists at others; and that odors can also function as enhancers, painting a much more complex picture of how odor sensing leads to perception of mixed odor blends than a simple combinatorial code. One possible value of this effect is that by modifying the strength of the signals detected within the periphery to enhance or suppress different components of a mixture, the dynamic range of the sensory system could be effectively increased. This model, illustrated in Figure 63, shows that if responses to individual odors generate an expected identifiable odor-specific code, then a simple summation or maximum projection of the codes of multiple odors, as in a blend, could quickly fill up the representation of the summed receptors (Model 1). This effect could make numerous mixtures of different odors indistinguishable from one another. The small changes in the combined code resulting from the effects of suppression and enhancement bestows a uniqueness on the combined code for the mixture (Model 2). In some cases, this effect may interfere with the ability to identify every odor component within a blend, but would instead provide the mix with a recognizable identity. To relate this model to our data, we note that measured cell response profiles shown in Figure 39 (cell (i) and cell (v)), correspond to the presumptive response patterns R2 and R8 in the proposed model.
Figure 63. Diversified coding capacity through modulation. Two conceptual models are shown to contrast their robustness in odor mixture coding. Non-modulation model (left): Suppose odors X, Y and Z (all monomolecular compounds) can each activate a subset of odorant receptors. In this model, mixing odor Y with odor X would recruit two more receptors but adding Z will not produce a different perception, although its response profile only partially overlaps with X and Y. Modulation model (right): In this model, all receptors are subject to modulation in addition to their activation profiles. Under one possible circumstance (similar to what we have observed), mixing odors X and Y results in the inhibition of receptor 2 and the enhancement of receptor 8. Adding odor Z into the mixture inhibits receptor 5 and enhances receptor 7. As a result, the sparsity is increased due to inhibition and the spectrum of odor coding is expanded through enhancement. Together these serve to increase the robustness of pattern detection as a mechanism of perception. This model also implies that ‘silent’ receptors (R7 and R8 in this case) might be as important as the activated ones in pattern recognition of an olfactory object.

Although humans possess an estimated 400 unique olfactory receptors, there are orders of magnitude higher numbers of potential ligands (i.e., odors). Making conservative estimates that any given odor molecule can activate 3-5 unique receptor types at a medium level of concentration, then a blend of just 10 odors could occupy as many as 50 receptors, more than 10% of the family of human receptors [143, 144]. The situation is worsened if some of these receptors have overlapping sensitivities, which will result in fewer differences between two blends of 10 similar
compounds. The number of available unoccupied receptors is further reduced with the addition of each new component, eventually saturating the system and making it impossible to discriminate between complex blends, nor identify any components within the blends. The modulatory actions we report here could ameliorate this problem, as detailed above and in Figure 63, by providing additional receptor patterns for complex blends to occupy. This model suggests that higher brain regions may utilize pattern recognition as an alternative to either combinatorial or analytical coding strategies.

Theoretically, odor responses in the olfactory bulb should largely correlate to those in the epithelium because glomeruli are essentially OSN axon terminals, although complex information processing happens at both pre- and post-synaptic levels [145, 146]. In two preprinted studies, in vivo glomerular responses to odor mixtures were recorded with two-photon imaging; both inhibition and enhancement were observed, consistent with our findings in the epithelium [147, 148]. However, it is much more difficult to predict the outcome of the modulation effects at higher brain centers due to the lack of discernable patterning or topographic arrangement of inputs from olfactory bulb to piriform cortex and other brain areas [92-94].

The psychophysical effects of mixing two odors have been well documented and characterized. It has long been known that the overall intensity of any binary mixture is generally weaker than the linear sum of those of the unmixed components, a phenomenon termed hypoadditivity or compensation [110, 115]. In addition to this non-specific effect, odor masking has also been frequently observed, where the smell of one odorous compound can be weakened by certain other compounds in a mixture [110-114]. This is also a common tool for perfumers; for example, isoraldeine (also known as γ-methyl ionone) and many other compounds have been used as masking agents [149, 150]. Enhancement is relatively rare, but has been occasionally reported
in behavior studies [151, 152]. Although these psychophysical phenomena are often attributed to higher centers, evidence suggests that the epithelium is also involved. In particular, early studies have shown that odor masking is more prominent in a physical odor mixture than a dichroic mixture [110], and that perceptual response changes were correlated to modulation effects in the periphery [118]. It has also been shown that odor recognition can be configural, consistent with our pattern recognition model [153, 154].

In other non-chemosensory systems (vision, audition, somatosensory, etc.) there are no demonstrated cases of a stimulus activating one receptor/detector and inhibiting another. In these systems, complex interactions among stimuli occur at higher levels of processing, e.g., red-green opponency in retinal ganglion cells. A nearly similar instance of peripheral modulation occurs in the auditory system where the delivery of two tones of particular frequencies can set up interference waves on the basilar membrane (known as two tone and missing fundamental illusions) [155]. However, these are entirely mechanical processes and do not involve physiological responses of the primary sensory neurons. Olfaction then appears unique in utilizing stimulus-induced complex activity starting at the level of primary sensory receptors. This unusual complexity at such an early level of sensory discrimination raises crucial questions about the similarity or dissimilarity of higher olfactory processing to that found in other sensory systems. There is thus strong motivation to consider alternative coding strategies for olfaction that are distinct from those identified in other sensory systems.

This chapter and part of Chapter 4 have been published as Xu et al., 2020 [156]. This study was conducted in collaboration with Dr. Elizabeth Hillman and her PhD student at the time, Dr. Wenze Li, who collected data together with me and provided support in SCAPE microscopy and
hardware. Drs. Venkatakaushik Voleti and Dong-Jing Zou also contributed to this study. For full details of author contribution, see Xu et al., 2020.
Chapter 6

DISCUSSION AND FUTURE WORK

The aim of this thesis is threefold. First, this thesis has demonstrated a way of understanding peripheral olfactory coding through the application of medicinal chemistry. It further shows that peripheral odor discrimination is primarily driven by the molecular topological properties of odors rather than their chemical properties. Second, this thesis has shown that the commonly used perfumery additive, Hedione, can act as a weak antagonist of many other odors at the receptor level. Following experiments have identified additional compounds (such as Fructalate) that could elicit Hedione-like modulation effects, suggesting that a variety of odor compounds may act as modulators. This hypothesis has been further extended to the third aim, where large-scale neuronal recordings have revealed widespread receptor-driven modulation in odor mixtures. This finding leads to a previously underappreciated fact that virtually any odor in a mixture may act as an agonist or antagonist, depending on the receptors and presence of other molecules in the blend. We have also found evidence of allosteric enhancement, a rarely observed phenomenon in ClassA GPCRs. In addition, this thesis has also demonstrated the application of the SCAPE microscopy, a potent and versatile imaging technique. The novel findings and technology development lead to multiple future directions, as discussed below.
6.1. **Continuation of the Hedione project**

Regardless of the interesting findings about the Hedione effect, current data are still preliminary and need to be repeated in a larger scale. Moreover, it remains unclear whether the peripheral modulation effects are indeed the cause of perceptual change. This question can be tested through human behavior experiments. Specifically, a leading odor (citral for example) will be delivered individually and then as a mixture with Hedione (Table 1, conditions 1-2). In one case the mixture will be delivered to one nostril (physical mixture) and in another case the two odors will be delivered simultaneously, but to different nostrils (dichorhinic mixture). We predict that the physical mixture, where interactions between odors and receptor cells can occur will be perceived differently than the dichorhinic mixture where there is no interaction at the peripheral level. If they are not perceived differently, then receptor interactions should have no effect on perception. A simplified version of odor delivery scheme is shown in the table below (Table 6).

<table>
<thead>
<tr>
<th>Condition#</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Left</strong></td>
<td>C</td>
<td>C+H</td>
<td>C</td>
<td>air</td>
<td>H</td>
<td>air</td>
<td>air</td>
<td>H</td>
<td>air</td>
</tr>
<tr>
<td><strong>Right</strong></td>
<td>H</td>
<td>air</td>
<td>air</td>
<td>H</td>
<td>C</td>
<td>C+H</td>
<td>C</td>
<td>air</td>
<td>air</td>
</tr>
</tbody>
</table>

*Table 6. Schemes for odor delivery.* Conditions 1-2 compares the effects of Hedione in dichorhinic mixture (#1) and in physical mixture (#2) in the left nostril; conditions 3-4 make the same comparison in the right nostril.

6.2. **Characterizing odor responses at single receptor level**

The mammalian odorant receptors comprise a very large gene family and their amino acid sequences show a considerable degree of variability. It remains unclear how structurally different odorants can activate the same OR, or how the same odorant molecule can activate different ORs and now also function as an antagonist, suppressing the responses evoked by other odorants. To
fully understand odor interactions in a mixture, thorough investigations at the single OR level would be highly illuminating. Many new research tools have been developed and are readily available for investigating odor interactions, specifically at two mouse ORs: M71 and M72. These ORs are highly similar differing by only 11 amino acids. Acetophenone and its derivatives are known to differentially activate both ORs [26, 157, 158]. Crossing the M71 (or M72)-RFP gene-targeted mice with the OMP-Cre/GCaMP6f line will allow us to use SCAPE microscopy to investigate and compare odor interactions at these two related and well characterized receptors (red fluorescence positive), and at other unidentified ORs (red fluorescence negative).

6.3. Optimizing the data processing pipeline

In my project (as detailed in the previous chapters), a 75s-long imaging session at 2VPS will generate ~20GB data consisting of 150 volumetric files. An experiment typically contains 15-20 sessions, and the data will only make sense when all the sessions are concatenated and analyzed together. This huge amount of data has imposed unique challenges in handling SCAPE data. Accordingly, I will continue seeking advanced computational tools and utilize them to optimize the data processing pipeline.
References


Appendix A: Supplementary Materials

A.1 CAD for mouse hemi-head perfusion chamber

Figure 64. CAD drawing of mouse hemi-head perfusion chamber.
A.2 SCAPE imaging of mouse olfactory epithelium activities

GCaMP6f fluorescence of intact olfactory epithelium (turbinate IIa-III) was recorded in response to a mixture of acetophenone, benzyl acetate and citral (100μM each). This video has been uploaded as a separate file for viewing.
Appendix B: Publications and Presentations Related to this Thesis

B.1  Peer reviewed publications


B.2  Conference presentations

1. Widespread receptor-driven modulation in peripheral olfactory coding.

   **Xu, L., Li, W., Voleti, V., Zou, D. J., Hillman, E. M. C. & Firestein, S.**

   Society for Neuroscience Annual Meeting. **Poster Presentation.**

   Chicago, Oct. 2019

2. Native olfactory sensory neuron imaging with swept confocally-aligned planar excitation (SCAPE) microscopy.

   **Xu, L., Li, W., Voleti, V., Hillman, E. M. C. & Firestein, S.**

   Society for Neuroscience Annual Meeting. **Poster Presentation.**

   San Diego, Nov. 2016