Chem. Senses 35: 323–333, 2010
doi:10.1093/chemse/bjq022
Advance Access publication March 8, 2010

Dummies versus Air Puffs: Efficient Stimulus Delivery for Low-Volatile Odors

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Accepted February 4, 2010

Abstract

Aiming to unravel how animals perceive odors, a variety of neurophysiological techniques are used today. For olfactory stimulation, odors are commonly incorporated into a constant airstream that carries odor molecules to the receptor organ (air-delivered stimulation). Such odor delivery works well for odors of high volatility (naturally effective over long distances) but less or not at all for low-volatile odors (usually only received at short range). We developed a new odor stimulation technique especially suited for low-volatile odors and compared it with conventional air-delivered stimulation using 2 neurophysiological approaches. Odor-loaded dummies were moved into close vicinity of the receptor organs on the antenna of the Florida carpenter ant *Camponotus floridanus* (dummy-delivered stimulation). Neuronal activity was monitored either at receptor neuron level using electroantennography or in the first olfactory neuropile, the antennal lobes, using calcium imaging. We tested 3 odors of different volatility: *C. floridanus*' highly volatile alarm pheromone undecane, its low-volatile trail pheromone nerolic acid, and an even less volatile, behaviorally active C23 alkene, cis-9-tricosene. For low-volatile odors, dummy-delivered stimulation was particularly efficient. We conclude that dummy-delivered stimulation is advantageous compared to the commonly used air-delivered stimulation when studying an animal's detection and processing of low-volatile odors.

Key words: ants, calcium imaging, *Camponotus floridanus*, electroantennography, neurophysiology, odor stimulation

Introduction

Odors are a major source of information for most animals and particularly insects heavily rely on chemical signals and cues for communication and orientation (Touhara and Vosshall 2009). Mating partner attraction in moths, where as little as a few pheromone molecules emitted by a distant female may elicit a behavioral response of a male, is an amazing example how extremely sensitive insect olfactory systems can be (Fabre 1900; Hildebrand 1995; Kaissling 2004). In face of danger to their colony, social insects like ants, bees, termites, or wasps often use pheromones to alert colony members in order to mount an effective defense (Butler 1609; Hölldobler and Wilson 1990; Landolt et al. 1998; Pasteels and Bordereau 1998; Schmidt 1998). The high volatility of sex pheromones in moths and alarm pheromones in social insects ensures efficient transport over long distances. For many insects, however, the chemical environment in close vicinity is of paramount importance and at short distances odors of low volatility become increasingly important. For several insect species, long-chain alkanes and alcohols of leaf cuticular waxes have been shown to be used for host plant selection (Müller and Riederer 2005). European beewolfs use a long-chain alcohol on the honeybee's cuticle for prey recognition (Herzner et al. 2005). Even tiny amounts of long-chain hydrocarbons left on plant surfaces (footprints) are used as cues, for example, by a parasitoid wasp for prey location (Rostas and Wölfling 2009) or by bumblebees to assess whether a potential food source has been recently visited by other bees (Wilms and Eltz 2008). Honeybees release several low-volatile semiochemicals during waggle dancing that have been shown to modulate recruitment (Thom et al. 2007). Ants are well known for laying foraging trails, and often low-volatile pheromones are used for long-term marking of trails (Moser and Silverstein 1967; Hölldobler and Wilson 1990). Another example illustrating the importance of low-volatile odors are complex mixtures of long-chained hydrocarbons on the

cuticle that are used as nestmate recognition cues by probably all social insects (Howard and Blomquist 2005; Leonhardt et al. 2007). The difference between olfaction and contact chemoreception (gustation) diminishes when insects receive odors of low volatility and stimulus delivery for neurophysiological experiments becomes increasingly challenging.

Commonly, air-delivered stimulation is used for stimulus delivery in neurophysiological experiments on olfactory systems of invertebrates (Anton and Hansson 1996; Joerges et al. 1997; Galizia et al. 1999; Sachse et al. 1999; Kleineidam et al. 2005; Hillier et al. 2006; Sandoz 2006; Silbering and Galizia 2007; Zube et al. 2008). Here, an airstream is directed over carrier material loaded with odor. Odor molecules diffuse into headspace, that is, they evaporate, dependent on the odor's vapor pressure, which in turn depends on the substance's volatility and the ambient temperature. The molecules are then carried away by the airstream to arrive at a distant receiver in packages (bulk flow). Odors of high volatility evaporate to a great extent and odor concentration in the bulk flow packages is relatively high, which allows odor detection in most instances. Odors of low volatility, however, evaporate to a minor degree and only few molecules are carried away by the airstream. In this case, odor concentration in the packages possibly is too low for detection. To circumvent this problem, we developed a new odor stimulation technique for neurophysiological experiments, which does not rely on an airstream to carry the odor to the receiver. Instead, we moved the odor source itself (an odor-loaded dummy) into close vicinity of the receptor organs in order to transiently increase the number of odor molecules right at the receiver site, that is, the effective concentration at the antenna (dummy-delivered stimulation). Such dummies are commonly used in behavioral experiments where the control of stimulus onset is less crucial and the insects are allowed to contact the dummy. However, in an earlier study, we could show that even hydrocarbons of very low volatility can be detected without physical contact (Brandstaetter et al. 2008). For the majority of neurophysiological experiments, contact stimulation will cause massive stimulus artifacts or may even ruin the preparation, and thus contact-free and time-controlled stimulation is desirable.

In this study, we compared air- and dummy-delivered stimulation using 2 different neurophysiological approaches. We first tested whether dummy-delivered stimulation is suited for a simple neurophysiological technique: electroantennography. Antennae of the Florida carpenter ant (*Camponotus floridanus*) were stimulated with 2 odors of high behavioral relevance: highly volatile alarm pheromone undecane and the low-volatile releaser component of the trail pheromone nerolic acid (Haak et al. 1996). In a second approach, we measured calcium signals of antennal lobe (AL) projection neurons in intact ants to evaluate whether air- or dummy-delivered stimulation is more efficient for 3 odors of different volatility: highly volatile alarm pheromone undecane, low-volatile trail pheromone nerolic acid, and a behaviorally active hydrocarbon of very low volatility, cis-9-tricosene.

Materials and methods

Animals

Colonies of the Florida carpenter ant *C. floridanus* have 1 single-mated queen (Gadau et al. 1996) and reach colony sizes of over 10 000 individuals. Queens of *C. floridanus* were collected in Florida, at Florida Keys after the mating flight in July 2003. For our experiments, a colony with a founding queen (collected by A. Endler and S. Diedering) was kept in the laboratory in an artificial plaster nest at a constant temperature of 25 °C and 50% humidity (12/12 h photoperiod) and provided with artificial diet (Bhatkar and Whitcomb 1970), honey water, and dead cockroaches (*Nauphoeta cinerea*) twice a week and water ad libitum. At the time of the experiments, colony size was approximately 3000 ants, and only large workers (head width > 3 mm) were used.

Odors

Odors of different volatility were used for stimulation: highly volatile alarm pheromone undecane (Sigma Aldrich), low-volatile trail pheromone (releaser component) nerolic acid (Cardiff Chemicals), and an even less volatile behaviorally active C23 alkene, cis-9-tricosene (Aldrich Chemical Company, Inc.). Our classification of high, low, and very low volatility is based on the corresponding vapor pressures of the odors used, as indicated in Table 1. All odors were diluted to various concentrations with custom-made distilled hexane. All neurophysiological experiments were conducted at a controlled room temperature of 25 °C.

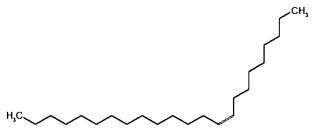
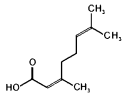
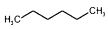
Air-delivered stimulation

A constant and moistened airstream of 1 L/min was produced by 2 independent flow controllers (VC-2LPM, Alicat Scientific), both set to 0.5 L/min. Only 1 of the 2 flow channels was used for stimulation during the experiments. A 1-s odor puff could be applied into the constant airstream by a computer-controlled solenoid valve, which allowed switching of the flow channel through a plastic cartridge (1 mL) containing a small filter paper (1 cm^2) loaded with odor. A quantity of 10 μL of diluted odor was applied onto the filter paper and for at least 4 min the solvent was allowed to evaporate before closure of the cartridge.

Dummy-delivered stimulation

Dummies served as a carrier for an odor. In principle, a broad range of different materials and forms can be used. Preferably, a dummy should possess an inert surface to allow easy cleaning between stimulations. In our electroantennography experiments, cylinders of brushed aluminum

Table 1 Odors

Odor	Volatility	Predicted vapor pressure (in mm Hg at 25 °C)	Chemical structure
cis-9-tricosene	Very low-volatile	3.16×10^{-6}	
Nerolic acid ([2Z]-3,7-dimethylocta-2,6-dienoic acid)	Low-volatile	3.73×10^{-4}	
Undecane	Highly volatile	5.64×10^{-1}	
Hexane (solvent)	Extremely volatile	151	

Classification of volatility, predicted vapor pressure, and chemical structure (according to www.chemspider.com).

(12×4 mm) were used. In calcium imaging experiments, we used magnetic stir bars (8×3 mm), which can be easily exchanged and cleaned between stimulations. The dummies were mounted on a holder connected to a bowden cable and could be moved into close vicinity of the antenna (distance ≤ 5 mm) by a computer-controlled servo motor (FS-501MGBB [calcium imaging] or RS-101MGB PRO-Line [electroantennography], Conrad Electronic SE). The dummy covered the initial distance of 12.5 cm to the antenna within 500 or 750 ms (FS-501 and RS-101, respectively) and stayed for a stimulation period of 1 s in calcium imaging and 1.6 s in electroantennography before being drawn back (Figure 1 and Supplementary Figure 5). Prior to stimulation, 20 µL of diluted odor was applied on hexane-rinsed dummies using a hexane-rinsed Hamilton syringe (Hamilton Company), and for 4 min, the solvent was allowed to evaporate.

Electroantennography

An antenna of a *C. floridanus* worker was cut at the base of the flagellum, and the last flagellar segment was carefully scarified in order to improve electrical contact between the antenna and the electrode. The flagellum was mounted between 2 chlorinated silver electrodes using a small amount of electrode gel (Spectra 360 Electrode Gel, Parker Laboratories, Inc.). The electrodes were connected to a 10-fold amplifier (Neuroprobe Amplifier Model 1600, A-M Systems, Inc.) and further amplified 100-fold and band-pass filtered (0.5–1 kHz) using a custom-made differential amplifier. The analog signal was then digitized using an A/D converter (NI USB-6211, National Instruments Germany GmbH), low-pass filtered (4 Hz), and analyzed with LabVIEW software (NI LabVIEW 8.2, National Instruments). The preparation was shielded with a Faraday cage to reduce electric noise.

The electroantennogram recordings were used to investigate whether dummies are suited for stimulation in a simple and relatively insensitive neurophysiological experiment. Undecane and nerolic acid at a dilution of 10^{-3} were applied via dummy-delivered stimulation, whereas pure solvent was used as control stimulus. For data analysis, data from the same preparation were pooled for each odor and the maximal amplitudes of the mean response curves during stimulation were calculated for each tested odors.

Calcium imaging

Large workers were immobilized by briefly cooling them on ice for a few minutes and then tethered in a custom-made Plexiglas stage using soft dental wax (surgident periphery wax, Heraeus Kulzer). A small window was cut in the head capsule with a piece of razorblade attached to a blade holder (Fine Science Tools GmbH) to access the brain and the site of dye application. Tracheae and glands were carefully moved aside with Dumont tweezers, and a sharp glass electrode was used to penetrate the tissue of the lateral protocerebrum, dorsolaterally to the vertical lobe of the right mushroom body. Subsequently, another sharp glass electrode coated with Fura-2 dextran (potassium salt, 10 000 MW, F3029, Molecular Probes) dissolved in 2% bovine serum albumin solution was inserted in the same region, aiming for the uniglomerular projection neurons of the median and the lateral antennocerebral tracts. The window in the head capsule was closed with the cut piece of cuticle, and the animals were kept in darkness and moistened air for a staining period of 6–8 h. Prior to imaging, antennae and

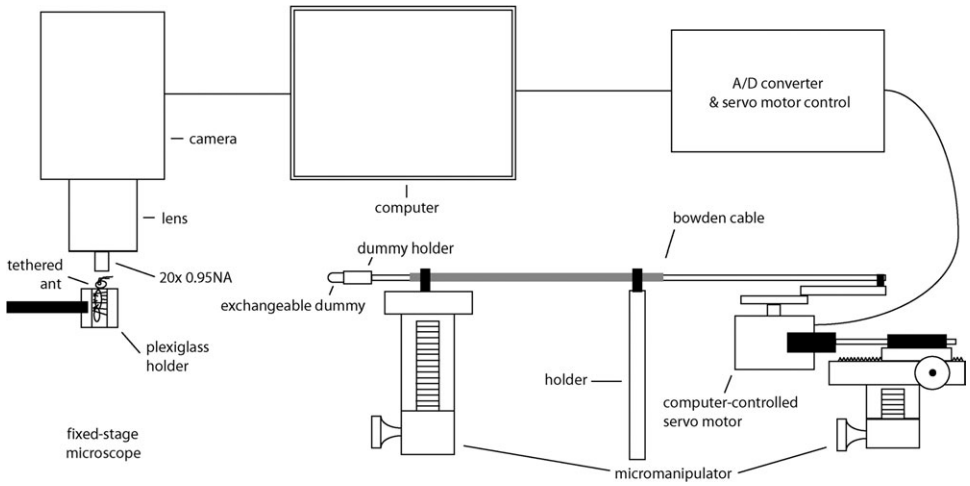


Figure 1 Dummy-delivered stimulation setup. An exchangeable, odor-loaded dummy is mounted on a dummy holder attached to a bowden cable. The dummy can be moved back and forth via a computer-controlled servo motor, and the exact dummy position is regulated via micromanipulators. For calcium imaging, an ant is tethered in a plexiglass holder under a fixed-stage microscope (imaging hardware not shown). See Supplementary Figure 5 for an animated drawing.

mandibles were fixed with wax, and the window in the head capsule was enlarged to access the right AL. Glands and trachea were carefully removed and the esophagus was pulled out of the head capsule to prevent movement of the brain during data acquisition. Hemolymph above the brain was removed and substituted by 2-component adhesive (KWIK-SIL, World Precision Instruments Germany) to further prevent movement and desiccation of the brain. We prepared 260 workers of which 152 (58.5%) showed bright staining of projection neurons in the AL.

Calcium imaging experiments were performed using an Olympus imaging system (Cell R v2.5, Olympus Imaging Europa GmbH) with an upright epifluorescent microscope (BX51WI; with filter set UM2FUR) equipped with an LD ×20 water-immersion lens (XLUMP, NA 0.95). Epifluorescent illumination was provided by a 150-W xenon light source (MT20, with excitation filters for 340 nm and 380 nm). The focal plane within the AL was adjusted to a depth of 30–40 µm below AL surface using a piezo-driven nanofocusing system (PIFOC P-721, PI). For each stimulus, a series of 40 double frames was recorded with an air-cooled CCD camera (model 8484-03G, Hamamatsu Photonics) at a sampling rate of 4 Hz. An 8×8 on chip binning resulted in an image pixel size of 2.58×2.58 µm. Exposure times ranged from 4 to 36 ms for the first frame at 340 nm and 1–12 ms for the second frame at 380 nm. Imaging data were analyzed by calculating the ratio of fluorescence intensity in the images taken at 340 and 380 nm excitation for each pair as: $R = F_{340}/F_{380}$ and subsequently applying an $N \times N$ filter (3×3 pixels with 3 iterations) to reduce noise. Autofluorescence and stained neurons caused inhomogeneous fluorescence images (background fluorescence). By subtracting the average ratio image of frames 1–17 (F_{ref}) from each ratio image (F_i) and dividing the result by F_{ref} ($\Delta F/F$), the background fluorescence was set to zero prior to stimulation.

Air- and dummy-delivered stimulation was controlled by the imaging software and started after double frame 20 (5 s). As a test stimulus for functionality, we presented air-delivered nerolic acid at a dilution of 10^{-5} and measured neuronal activity in about 11.5% of all preparations.

There is no standard AL atlas available for *C. floridanus* and, consequently, calcium signals could not be assigned to identified glomeruli. The AL of *C. floridanus* consists of approximately 450 glomeruli (Zube et al. 2008), which are smaller than, for example, *Apis mellifera's* 165 glomeruli (Arnold et al. 1985), and it contains no prominent landmark glomeruli that would allow orientation. Hence, unlike for the honeybee AL, neuronal activity cannot be analyzed across specimens but only within each single preparation. Our data evaluation has been designed according to these constraints.

Evaluation of the stimulation method

First, we investigated how low-volatile nerolic acid is represented in the AL upon air- and dummy-delivered stimulation. We used a high concentration (dilution of 10^{-1}) of nerolic acid and selected regions of interest (ROIs) that matched the neuronal responses within the AL. Activity patterns as well as their mean kinetics (temporal dynamic of activity) were compared across the 2 different stimulation techniques. Threshold for neuronal activity was defined as 40% of the maximal response (Zube et al. 2008). The activity patterns we measured were largely overlapping when air- and dummy-delivered stimulation was compared within 1 specimen. However, we found slight differences in the activity patterns that we first considered as contamination of the air-delivered stimulation apparatus. Even after exchanging

and carefully cleaning the air-delivered stimulation apparatus, the patterns of activity consistently remained largely overlapping but with minor differences ($N = 5$). These differences may be caused by residues of the solvent hexane and/or other contaminants resulting in neuronal interactions between glomeruli. Additionally, differences in effective concentration due to different stimulation efficiency of the 2 stimulus delivery techniques may also result in slight variation of the neuronal patterns. Possible causes for the found differences in odor representation will be discussed in detail below. The following evaluation method was carefully designed to compensate for minor differences in the neuronal representation.

In order to evaluate whether dummy-delivered stimulation is better suited for stimulation with low-volatile odors than air-delivered stimulation, we compared the detection level for 3 odors of different volatility with either air- or dummy-delivered stimulation. We used highly volatile undecane as reference odor at a relatively low odor concentration (dilution of 10^{-5}), known from previous experiments to be in a physiologically relevant range (Zube et al. 2008). Filter papers for air-delivered stimulation and dummies were loaded with different volumes of diluted odor at a specific ratio (undecane: dilution of 10^{-5}; air-delivered 10 μL, dummy-delivered 20 μL). We selected this loading ratio based on pilot experiments in order to ensure that undecane can clearly be detected with calcium imaging when either of both stimulation techniques is used. Pure solvent was used as a control stimulus and we assured that the control-corrected fluorescence change (relative intensity change) for both stimulation techniques was in the same range. We then used the same loading ratio for the low-volatile odors and tested whether detection level was reached with air- and dummy-delivered stimulation (nerolic acid: dilution of 10^{-5} and *cis*-9-tricosene: dilution of 1.25×10^{-3}; both odors: air-delivered 10 μL, dummy-delivered 20 μL). In a previous behavioral study, we could show that ants can detect *cis*-9-tricosene over a short distance when it is presented on dummies at a dilution of 1.25×10^{-3} (Brandstaetter et al. 2008), and therefore we used this odor concentration in the present test series. If dummy-delivered stimulation is better suited for low-volatile odors than air-delivered stimulation, it is expected that low-volatile odors can be detected when presented on dummies but not when delivered via airstream. Accordingly, the difference between the relative intensity changes upon air- and dummy-delivered stimulation should increase with decreasing volatility.

To determine whether detection level was reached, we compared the fluorescence change of air- and dummy-delivered odor stimulation with the appropriate control stimulations within specimens in a test series of several animals. For each tested odor, 4 ROIs were selected in each single preparation. 2 of the odor-specific ROIs were selected on the basis of the 2 activity spots showing strongest neuronal activity in response to air-delivered stimulation, the other 2 odor-specific ROIs were selected accordingly on the basis of dummy-delivered stimulation. This was done in order to assure that both stimulation techniques were equally represented in the evaluation in case partially different activity patterns were measured upon air- and dummy-delivered stimulation. The mean fluorescence change during stimulus presentation was calculated for each ROI, and subsequently, the mean fluorescence change of the 4 odor-specific ROIs was calculated for both stimulation techniques. The fluorescence changes for each odor and the respective control stimulation were compared pairwise for both air- and dummy-delivered stimulation using paired *t*-tests (Statistica 7.1, StatSoft).

Results

Electroantennography

Electroantennography provides an easy way to test dummy-delivered stimulation in a simple neurophysiological approach. We measured clearly detectable responses to dummy-delivered odor stimulation in 4 antennal preparations. We performed repeated measurements within those preparations, resulting in a total of 13 recordings in response to nerolic acid, 8 recordings in response to undecane, and 14 recordings in response to control stimulation (solvent only). Repeated measurements within specimens were pooled, and mean responses for the 4 preparations were calculated. The mean response curves for dummy-delivered stimulation with undecane, nerolic acid, and solvent only (control) are depicted in Figure 2 and their maximal response amplitudes are given in Table 2. We used an odor dilution of 10^{-3}; this odor concentration is relatively high but within a concentration range also used with more sensitive techniques like calcium imaging technique (Zube et al. 2008).

Calcium imaging

Neuronal activity upon air- and dummy-delivered stimulation could be measured for different odors. We first compared the patterns of neuronal activity for air- and dummy-delivered nerolic acid stimulation at a dilution of 10^{-1} (Figure 3 shows an example of neuronal responses in 1 specimen). Both, air- and dummy-delivered nerolic acid stimulation resulted in pronounced and reproducible patterns of neuronal activity. We found largely overlapping areas of activity irrespectively whether air- or dummy-delivered stimulation was used. However, we found consistently small areas that were activated only upon air- or only upon dummy-delivered stimulation (Figure 3C–F). Dummy-delivered nerolic acid stimulation generally elicited stronger fluorescence changes than air-delivered stimulation, which is evident in the response kinetics, where the maximal fluorescence change is 3.7% upon dummy- and 2.3% upon air-delivered stimulation (Figure 3G,H). This indicates that reception of low-volatile odors like nerolic acid is facilitated

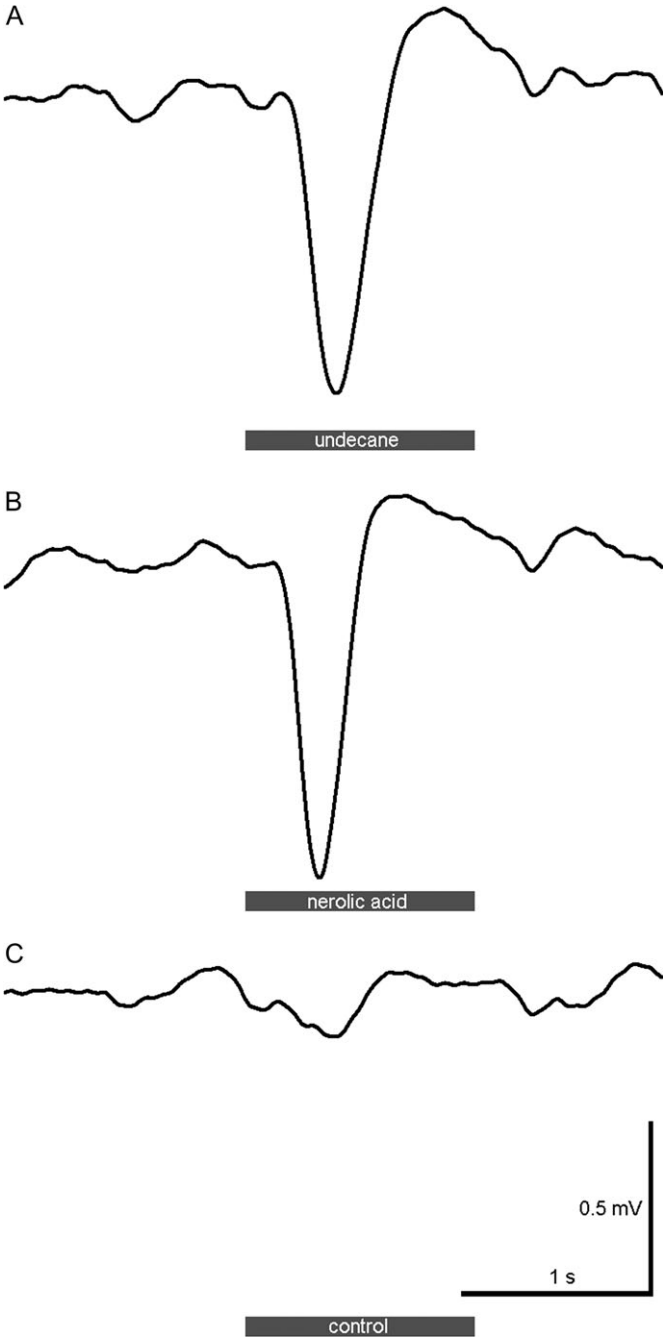


Figure 2 Electroantennography of dummy-delivered stimulation. Mean sensory responses of olfactory receptor neurons (amplitude: ~ 1 mV) were measured for both undecane **(A)** and nerolic acid **(B)**. Both odors were presented on dummies. No voltage response was measured when a control dummy (solvent only; **C**) was presented. A gray bar indicates the time the dummy was close to the antenna. All recordings were taken from 4 antennal preparations with repeated measurements. This resulted in a total of 13 recordings for nerolic acid, 8 recordings for undecane, and 14 control recordings (solvent only).

when dummies are used for odor presentation instead of stimulation via an airstream. We tested this hypothesis in the following experiment, and data evaluation was designed

Table 2 Electroantennography of dummy-delivered stimulation

Odor	Response amplitude (mV)	Standard error	Coefficient of variation
Undecane	−0.82	0.22	−54.7
Nerolic acid	−0.89	0.087	−19.5
Control (solvent)	−0.14	0.019	−26.8

Maximal amplitudes of mean response curves upon dummy-delivered stimulation (with standard error and coefficient of variation).

to compensate for minor differences in the neuronal activity patterns elicited by either air- or dummy-delivered stimulation.

To evaluate the 2 different stimulation techniques, we measured neuronal activity in response to air- and dummy-delivered stimulation using 3 different odors in 8–14 specimens (depending on the odor). As explained in "Evaluation of the stimulation method," we used lower odor concentrations than in the preceding experiment (dilutions for nerolic acid and undecane: 10^{-5}, for *cis*-9-tricosene: 1.25×10^{-3}). We then investigated the relative response intensities, that is, the control-corrected mean fluorescence changes, for the different odors (Figure 4).

Undecane stimulation resulted in a significantly higher fluorescence change than the control stimulation for both air-delivered (t = 2.70, DF = 13, P = 0.0183) and dummy-delivered stimulation (t = 4.48, DF = 13, P = 0.000620). The relative intensity change was in the same range for both stimulation techniques.

For nerolic acid, no significant difference in fluorescence change was measured for air-delivered nerolic acid compared to the control stimulation (t = 1.56, DF = 13, P = 0.143). In contrast to this, fluorescence change upon dummy-delivered nerolic acid stimulation was significantly higher than upon dummy-delivered control stimulation (t = 4.08, DF = 13, P = 0.00131).

Air-delivered *cis*-9-tricosene stimulation was not significantly different from air-delivered control stimulation (t = 0.571, DF = 7, P = 0.586). Although dummy-delivered *cis*-9-tricosene stimulation resulted in a relative intensity change comparable with that of dummy-delivered nerolic acid stimulation, no significant difference could be found in comparison with control dummy stimulation (t = 1.81, DF = 11, P = 0.0975).

In summary, comparisons within specimens show that nerolic acid is received better when presented on a dummy compared with stimulation via airflow. Relative intensity changes upon air- and dummy-delivered undecane stimulation were in the same range, whereas relative intensity changes upon dummy-delivered stimulation with low-volatile nerolic acid and very low-volatile *cis*-9-tricosene were apparently higher than upon air-delivered stimulation. For *cis*-9-tricosene, however, we found a very high variance

in the data, and this indicates that stimulation efficiency decreases when volatility is very low (vapor pressure at 25 °C < 10^{-6} mm Hg). Dummy-delivered stimulation in its current design appears, thus, especially efficient for odors of low volatility (vapor pressure at 25 °C of up to 10^{-4} mm Hg) but may also be suitable for highly volatile odors. Stimulation with odors of very low volatility remains challenging at this stage, but further technical improvement of dummy-delivered stimulation has the potential to improve stimulation efficiency even for very low-volatile odors.

Discussion

We present a new odor stimulation technique for neurophysiological experiments. Odor-loaded dummies were successfully used for stimulation in electroantennography and calcium imaging. Both undecane and nerolic acid elicited strong responses in both neurophysiological approaches when presented on dummies. We evaluated the efficiency of this new stimulation technique using calcium imaging and showed that dummy-delivered stimulation is better suited for presentation of low-volatile odors than air-delivered stimulation.

Electroantennography revealed that odor-loaded dummies can be used for stimulus delivery in a simple neurophysiological approach. Presentation of undecane and nerolic acid on dummies resulted in measurable summed potentials of around 1 mV amplitude, whereas no such response could be measured upon control stimulation (Figure 2). Dummy-delivered stimulation with low-volatile nerolic acid elicited strong responses in olfactory receptor neurons of the antenna with a low standard error and coefficient of variation in comparison with dummy-delivered stimulation with highly volatile undecane (Table 2). This indicates that dummy-delivered stimulation may be particularly well suited for stimulation with low-volatile odors, resulting in stable sensory responses. For a detailed evaluation of air- and dummy-delivered stimulation, we used calcium imaging, which is more sensitive and allows a more detailed analysis than electroantennography.

We compared the neuronal activity patterns upon either air- or dummy-delivered stimulation with low-volatile nerolic acid at a high concentration (dilution of 10^{-1}). We measured pronounced neuronal activity upon nerolic acid stimulation using either of both delivery methods and repeated stimulation resulted in reproducible patterns of neuronal activity. The patterns of neuronal activity elicited upon air- and dummy-delivered stimulation were largely overlapping with minor differences and the same was true when the same stimulus delivery technique was repeatedly used (Figure 3C–F). These differences might be explained by the high sensitivity of the olfactory system in ants (Zube et al. 2008). Even upon air-delivered control stimulation (Figure 3B), we repeatedly measured neuronal activity that was most probably caused by solvent residues. As neuronal activity in response to dummy-delivered control stimulation occurred much less persistently and less pronounced (data not shown), the solvent we used (hexane) apparently evaporates at a different rate from dummies than from filter papers used for air-delivered stimulation and remaining solvent may influence the neuronal representation. We cannot completely rule out further contamination of the air-delivered stimulation apparatus, although we put large efforts into eliminating contamination effects. However, the simple design and easy cleaning of dummies makes them less prone to contamination than air-delivered stimulation, which provides a substantial advantage. Regardless of whether solvent residues or contamination are detected in concert with air-delivered nerolic acid, the highly sensitive insect olfactory system integrates odor information by interactions between glomeruli via local interneurons, which consequently is reflected in minor changes of the neuronal representation (Lei and Vickers 2008; Silbering et al. 2008; compare ROI 4 in Figure 3G,H and black arrows in Figure 3C–F). Dummy-delivered nerolic acid stimulation resulted in a higher fluorescence change and, thus, higher neuronal activity than air-delivered nerolic acid stimulation (Figure 3G,H). This indicates that the effective concentration at the olfactory receptors on the antenna was higher for dummy- than for air-delivered stimulation. A difference in effective concentration influences the neuronal representation in the AL eliciting stronger neuronal responses when effective concentration is higher and this may lead to concentration-dependent changes in the spatial activity patterns as previously demonstrated in calcium imaging studies in the honeybee and ant (Sachse and Galizia 2003; Zube et al. 2008; compare ROI 3 in Figure 3G,H and gray arrows in Figure 3C–F). In conclusion, qualitative odor reception is largely similar, whereas quantitative odor reception differs supposedly as a result of different effective concentrations at the receptor neurons due to different stimulation efficiency of the 2 stimulus delivery methods. We tested this hypothesis experimentally and carefully designed the data evaluation to compensate for minor differences in the neuronal activity patterns.

To evaluate whether dummy-delivered stimulation is better suited for presentation of low-volatile odors than air-delivered stimulation, we measured neuronal responses in the AL upon air- and dummy-delivered stimulation with 3 odors of different volatility: undecane, nerolic acid, and *cis*-9-tricosene (Figure 4). Highly volatile undecane was used successfully with both stimulation techniques and elicited strong neuronal activity. We conclude that for odors of high volatility, both stimulation techniques are well suited. We used undecane as a reference odor and compared the detection levels for air- and dummy-delivered stimulation using low-volatile odors at the same loading ratio. For low-volatile nerolic acid at a low odor concentration (dilution of 10^{-5}), which is well in a behaviorally relevant range, the patterns of neuronal activity elicited by air- and dummy-delivered stimulation partially differed from each other and our data

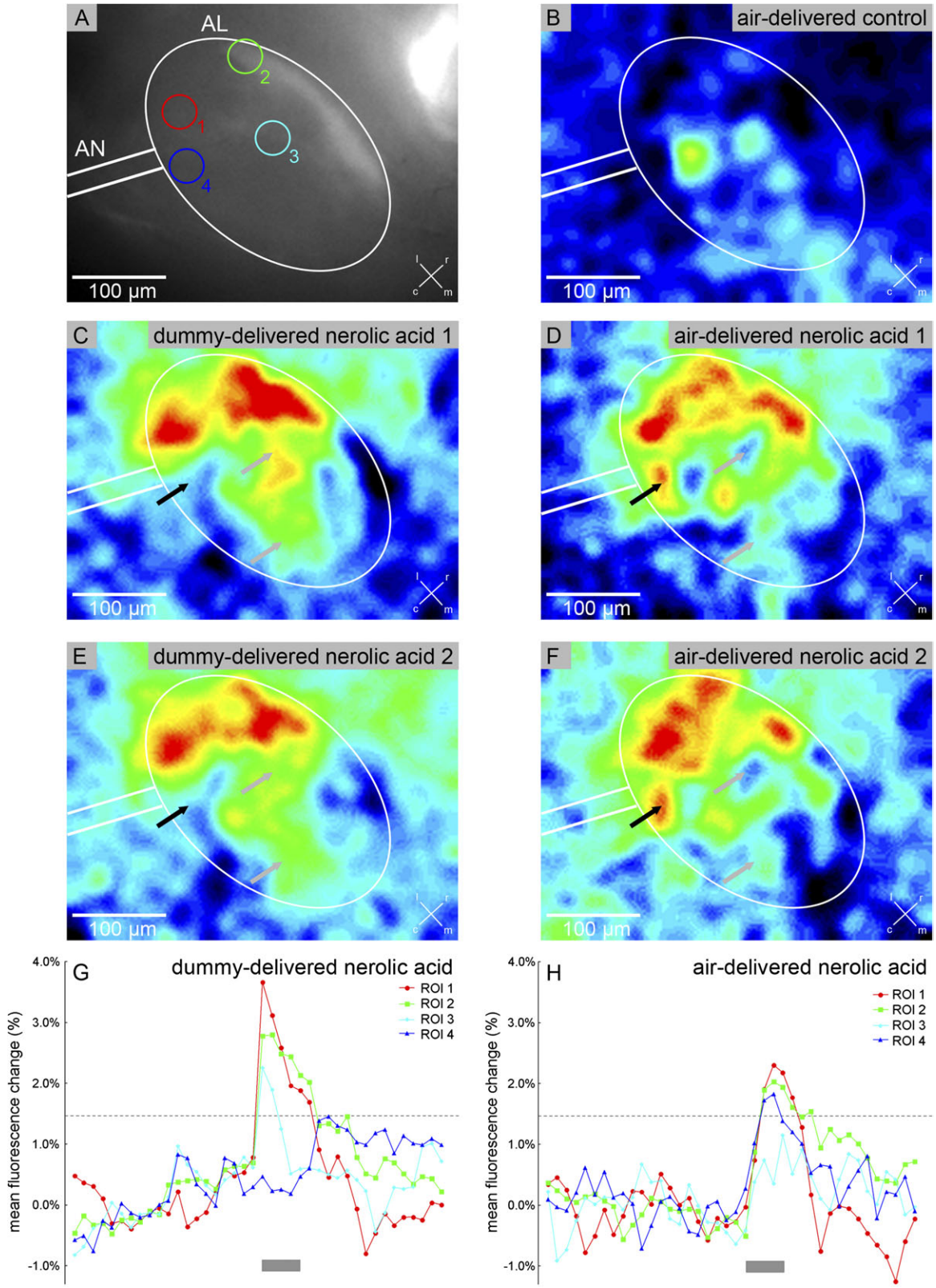


Figure 3 Calcium imaging: neuronal activity in the AL upon stimulation with nerolic acid at a high concentration (dilution of 10^{-1}). **(A):** Epifluorescent micrograph of the AL at 380 nm excitation wavelength. ROIs are indicated by colored circles. AN, antennal nerve; AL, antennal lobe; l, lateral; m, median; r, rostral; c, caudal. **(B–F):** False color coded recordings of neuronal activity in the AL 500 ms after stimulus onset (red indicates areas of high neuronal activity; note that in order to visualize the neuronal activity patterns, different intensity-coding color ranges were used for air- and dummy-delivered stimulation;

evaluation method compensated for slight differences in odor representation. Although relative intensity changes upon dummy-delivered stimulation were significantly above noise level, this was not the case upon air-delivered stimulation. We conclude from this that detection of low-volatile odors, like nerolic acid, is facilitated when dummies are used for presentation. In contrast to our experiment, air-delivered nerolic acid stimulation at the same concentration resulted in measurable responses in a previous study (Zube et al. 2008). In the present study, we used a more conservative measure in order to determine whether detection level was reached or not, which explains the differing results. We propose that dummy-delivered stimulation is more efficient than air-delivered stimulation for odors of low volatility because more odor molecules reach the antenna, and thus, the effective odor concentration is higher when presented on dummies compared with air-delivered stimulation.

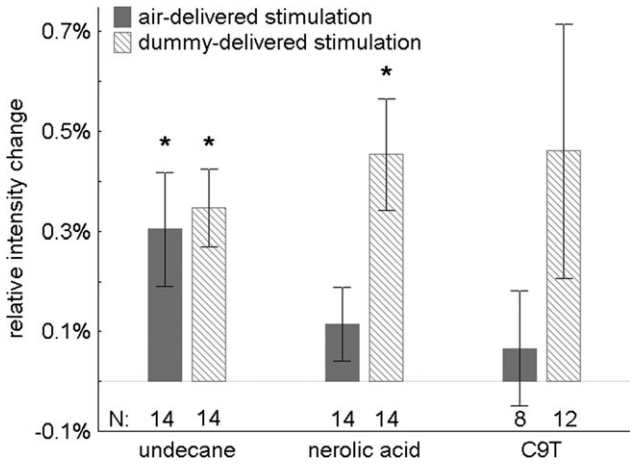


Figure 4 Calcium imaging: relative intensity change upon air- and dummy-delivered stimulation. Mean relative intensity change (i.e., control-corrected mean fluorescence change) is depicted in bars with standard error for highly volatile undecane, low-volatile nerolic acid, and very low-volatile *cis*-9-tricosene (C9T). Relative intensity changes, where the odor-induced fluorescence change differs significantly from control-induced fluorescence change in paired *t*-tests are marked with an asterisk. *N*: number of specimens tested per odor. For highly volatile undecane, both air- and dummy-delivered stimulation were above detection level and elicited significant neuronal responses. Low-volatile nerolic acid elicited significant neuronal responses only when presented on dummies. Dummy-delivered stimulation with C9T elicited a strong relative intensity change, however, there was no significant difference. For air-delivered stimulation relative intensity change decreases with decreasing volatility, whereas for dummy-delivered stimulation relative intensity change increases with decreasing volatility. At very low volatility, however, variation is very high and stimulation efficiency is, thus, substantially decreased.

In addition to the pheromones undecane and nerolic acid, we used a behaviorally active C23 alkene for stimulation in calcium imaging. *Cis*-9-tricosene has a very low volatility but has been shown to be detectable by free-moving ants in a behavioral assay if sufficient time is given (in that case 3 min) at 25 °C room temperature even if contact was not permitted (Brandstaetter et al. 2008). Neuronal activity for air-delivered *cis*-9-tricosene was negligible in all cases, whereas dummy-delivered stimulation sometimes worked very well, eliciting strong neuronal responses, and sometimes not at all, indicated by the high variance in relative intensity change upon dummy-delivered *cis*-9-tricosene stimulation (Figure 4). *Cis*-9-tricosene has a very low volatility (see Table 1), and we suspect that a combination of several, subtle factors has to be favorable to allow stimulation with this substance. First, room temperature was kept around 25 °C, however, small fluctuations in temperature close to the antenna may have influenced the diffusion of *cis*-9-tricosene in headspace. Second, positioning of the dummy was technically limited to a range of ±3 mm, and such small differences in the exact distance to the antenna may have prevented successful detection of *cis*-9-tricose. Third, it is not known where on the antenna the receptor neurons sensitive to long-chain hydrocarbons are located. It may well be that those sensilla are located only in restricted regions of the antenna, and for the detection of a low-volatile odor like *cis*-9-tricosene, an appropriate stimulation site for the dummy might be crucial. Stimulation efficiency with odors of very low volatility is not yet optimal, however, we are confident that technical improvements of our dummy-delivered stimulation technique have a great potential of enhancing this new stimulus delivery even further. More sophisticated positioning systems will allow the dummy to be positioned much closer to the receptor organ than in our current setup and means to increase the vapor pressure of very low-volatile odors will further push

images **C**–**F** are control-corrected). Air-delivered control stimulation **(B)** persistently elicited activity spots caused either by residues of solvent or contamination of the air-delivered stimulation apparatus. Repeated dummy- **(C** and **E)** and air-delivered **(D** and **F)** nerolic acid stimulation resulted in reproducible albeit not absolutely identical patterns of neuronal activity. Neuronal activity was wider spread upon dummy- than upon air-delivered stimulation (see gray arrows in **C** and **E**; compare ROI 3 in **A** and median parts of AL in **C** and **E**). A single area in the caudal part of the AL was activated upon air- but not upon dummy-delivered stimulation (see black arrows in **D** and **E**; compare ROI 4 in **A**). Otherwise the areas activated upon air-delivered stimulation corresponded to the areas activated upon dummy-delivered stimulation. **(G and H):** Kinetics of the fluorescence change (temporal dynamics of activity) of ROIs 1–4 (compare **A**) upon dummy- **(G)** and air-delivered **(H)** nerolic acid stimulation (mean fluorescence change of stimulation 1 and 2, respectively). For both stimulation techniques, a rise in fluorescence change above threshold for neuronal activity (dashed line) was measured upon stimulation, indicated by a gray box. ROIs 1 and 2 exhibit similar kinetics upon air- and dummy-delivered nerolic acid stimulation, although the fluorescence change was higher for dummy-delivered stimulation. This may be explained by a higher effective concentration at the receptor neurons upon dummy- compared with air-delivered stimulation. For ROI 3, neuronal activity was only detected upon dummy-delivered stimulation, whereas ROI 4 showed neuronal activity upon air- but not upon dummy-delivered stimulation. Activation of slightly different areas of the AL upon stimulation with air- or dummy-delivered stimulation may be explained by differences in effective concentration and different interaction of local interneurons due to residues of solvent and/or contamination of the air-delivered stimulation apparatus.

the limits closer toward investigating neuronal processing during contact chemoreception.

In conclusion, we present a new and easy-to-use technique for odor stimulation in neurophysiological experiments. Dummy-delivered stimulation proved to be particularly advantageous for the presentation of low-volatile odors. In addition to the technical improvement, dummy-delivered stimulation better resembles the natural situation of odor dispersal, for example, from a food source or a foraging trail. Thus, it better simulates close-range odor detection in a natural habitat compared with commonly used air-delivered stimulation.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Funding

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, Germany (SFB 554/A6). A.S.B. was supported by a grant of the German ''Excellence Initiative'' to the Graduate School of Life Sciences, University of Würzburg.

Acknowledgements

We thank Katrin Vogt for experimental support in electroantennography. We also thank Timothy McClintock and 2 anonymous reviewers for their valuable comments on an earlier version of the manuscript. The performed experiments comply with the current laws of the Federal Republic of Germany.

Author contribution: A.S.B. and C.J.K. conceived and designed dummy-delivered stimulation. A.S.B., W.R., and C.J.K. designed the experimental procedure. A.S.B. collected calcium imaging data and did the data analysis. A.S.B. and C.J.K. wrote the manuscript.

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