Sniffing cancer: Will the fruit fly beat the dog?

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Odours can be used to detect cancer

Diagnosing diseases by the smell and taste of bodily samples goes back to ancient history. While the smell of some infections or metabolic diseases has been used as a diagnostic tool for a long time, it has only recently been observed that cancer is also detectable by its specific scent.

The first reports about dogs sniffing cancer were anecdotal. A pet dog that was constantly sniffing at a lesion on its owner’s leg, even through her trousers, made the woman suspicious. When the dog tried to bite off the lesion, she went to a clinic to get medical advice. The histological examination revealed a malignant melanoma (Williams and Pembrooke, 1989).

Once it was known that cancer alters the emitted odour of a patient, research was intensified on the usage of odour profiles for diagnostic purposes. In particular, animals were found to be highly sensitive for detecting cancerous odours. Research on electronic noses for cancer detection was intensified as well and different types of electronic noses are now showing promising results.

The early detection of cancer is crucial for successful cancer treatment. Therefore, reliable, non-invasive screening methods are important. Here, an odour-based approach is a promising strategy, which holds great potential as a fast and cheap screening tool.

In this review, we look at how odours may be early markers for tumours, we report on the success stories using dogs and on an alternative, potentially routine approach using fruit flies instead.

Cancer cells produce unique metabolite compositions, including volatile organic compounds

Body odours mainly consist of small volatile organic compounds (VOCs) produced by cell metabolism. They distribute throughout the body in the circulatory system, and can emanate from the body via breath, urine, sweat or other bodily products.

Cancer cells have a fundamentally different metabolism compared to normal cells, not least due to their tendency to grow fast and in an uncontrolled manner (Tennant et al., 2010; Ward and...
Thompson, 2012). This extraordinary metabolism alters the metabolite composition in the cell and the cell’s surrounding medium and consequently the VOC profile. For example, cancer cells may produce cancer-specific metabolites, and/or shift the relative concentration of common metabolites. These changes are then reflected in the emitted odour profile of cancer cells.

When a tumour produces cancer-specific metabolites, there is potential for these be used as diagnostic markers. Much research has been performed on identifying these biomarkers and developing screening assays for these specific metabolites - such as the prostate-specific antigen (PSA) screen for prostate cancer. However, no such marker has been discovered for most other cancer types. Indeed, many tumours are currently only detected when they are already large enough to be visible with imaging techniques. By contrast, an altered odour profile in body samples, like breath, urine, sweat or blood, might already be detectable when the tumour is still small and in an early stage.

For an odour-based diagnostic approach it is also not crucial to know the exact identity of the cancer-specific metabolites and biomarkers, since such an approach rather relies on concentration shifts, than on single biomarkers.

Other diseases, including infections, also generate specific odour profiles which could be used for diagnosis (reviewed in Bijland et al., 2013). These odours may derive from the infected cells or from other parts of the body which may be affected by the sickness. It has even been suggested that the immune system creates an aversive body odour to keep healthy individuals at a distance and thus reduce further infections (Olsson et al., 2014).

**Chemical analyses (GC-MS) show differences in the VOC composition between healthy and cancer patients**

Several studies aimed at deciphering the chemical composition of the VOC profiles from cancer patients and healthy subjects have emerged. Different bodily samples were analysed in order to find potential differences for diagnostic approaches. However, the first results showed that already among the healthy population variability among VOC compounds is high: of more than 3000 VOCs currently known in breath samples, only 20-30 were found in all healthy subjects (Pauling et al., 1971; Phillips et al., 1999a). In breath of cancer patients
no single absolute marker molecule of cancer could be identified using gas chromatography and mass spectrometry (GC-MS), but a combination of 22 VOCs was found specifically in lung cancer patients, and a combination of 15 VOCs was specific for colorectal cancer patients (Altomare et al., 2013; O’Neill et al., 1988; Phillips et al., 1999b). These VOCs were predominantly alkanes, benzenes and their derivates. The main difference between healthy and cancer patients was in the abundances of the VOCs rather than in their identity.

Gas chromatography followed by mass spectrometry is a powerful approach for these analyses, because of its fairly high sensitivity and the possibility to identify single components in an odour mixture. However, the analysis needs highly specialized labs. Furthermore, instable odour components are potentially degraded by heat before being identified. Thus, faster screening methods which work at room temperature and analyse the odour mixtures as a whole are needed.

Dogs smell cancer successfully, also against variable background

The sense of smell of dogs is very accurate. With their fine noses they help in many situations where high olfactory sensitivity is required. Detection dogs are trained and used successfully to detect substances, such as explosives, drugs or blood, and have been employed in the daily police work for a long time already. They perform astonishingly well in finding even slightest traces of these substances. Dogs can also track people by their body odour and discriminate the body odour of different humans.

There has been a wave of investigations on the performance of dogs in detecting cancer (reviewed in Bijland et al., 2013; Boedeker et al., 2012; Lippi and Cervellin, 2012). Dogs sniffed e.g. melanoma tissue directly on the skin (Pickel et al., 2004), lung and breast cancer in breath (Ehmann et al., 2012; McCulloch et al., 2006), ovarian carcinoma in tissue and blood samples (Horvath et al., 2008, 2010, 2013), prostate (Cornu et al., 2011) and bladder cancer (Willis, 2004) in urine samples and colorectal cancer in breath and watery stool samples (Sonoda et al., 2011). These abilities are paralleled by the ability to detect many other diseases, such as diabetes and infections, a topic that is not covered in this review. Charities in various countries train dogs for the benefit of patients (e.g. http://medicaldetectiondogs.org.uk/, http://www.krebssuchhunde.at/1.html.

Dogs tell us about cancer using their behaviour

In order to be used as cancer detection dogs, dogs have to be trained beforehand with healthy and cancer samples. During the training procedure, the dogs are rewarded when they indicate a cancer sample correctly and ignore the healthy control samples. Then, in the test phase, they are presented with samples of patients and are asked to detect cancer samples. Thus, our knowledge about the animal’s judgement comes via the animal’s behaviour. However, sometimes, the mood of the dog may falsify the result. Moreover, the behaviour of the dog depends on the relationship between the dog and its trainer and the
exhibited behaviour has to be interpreted correctly by a human interpreter. Furthermore, professional cancer detection dogs have to be continuously trained to keep the high performance. Thus, selection, training and maintenance of professional cancer detection dogs (and their trainers) are time-consuming and expensive.

**Animal olfactory systems are sensitive, universal and combinatorial**

At a first glance, it appears strange that a dog is able to smell human cancer. Did evolution give the dog a particular advantage, so that it has "cancer detection receptors"? We think not. Rather, it is the beauty of animals' olfactory systems which create the ability to encode millions of odours, including many odours that a species may never have encountered in evolution. How is that possible? And how can dogs discriminate odour mixtures by only minimal changes in the composition of single odour components? Several aspects are important here.

First, olfactory receptors in animals are generally **very sensitive** with respect to interacting with their odour ligands. Many can respond to their ligands at extremely low concentrations: a few odour molecules already elicit neural responses in the respective olfactory receptor neurons. The neurons then send the odour information to the brain for further processing.

Second, most olfactory receptors are **broadly tuned**, which means they respond to a wide range of different odours, including some that do not occur in the natural environment of the animal.

Third, odour coding is combinatorial. Olfactory receptors have different but overlapping receptive ranges of odorants to which they respond. As a result, an odorant usually activates several receptor types, creating a combinatorial ensemble response of activated (and inhibited) olfactory receptor neurons. This combinatorial pattern is unique for each odour (see Fig. 1). It is then the task of the brain to interpret the combinatorial pattern and categorise the odour stimulus. The importance of combinatorial coding is easy to grasp if you just think about the numbers: with 5 selective receptor neurons, an animal could smell 5 odour substances. However, 5 receptor neurons can create 31 on-off patterns ($2^{5}-1$). Imagine how many patterns a dog, with over 1000 receptor types, can encode: $2^{1000}$! It appears clear that this system is not limited by the dimensionality of the receptor neuron ensembles, but by variability, noise, and the readout capacity of the brain.

The capacity of a dog's nose to detect millions of odours is in strong contrast to the possible behavioural readout. The dog responds to a simple yes/no decision. But how advanced/large is the cancer? Is it of a particular kind? Possibly, the olfactory system of the dog has that information, but currently we cannot tell from its behaviour.

**Fruit flies allow direct access to physiology**

In order to circumvent the behavioural readout of dogs, while keeping the advantages of natural olfactory...
Figure 1: The dog’s olfactory periphery and the principle of combinatorial odour coding.

A) Schematic drawing of the dog’s olfactory periphery. The olfactory epithelium covers a large area in the nose and has a huge surface. In the olfactory epithelium olfactory receptor neurons (different types are coloured red, yellow, green and blue) express different types of olfactory receptors. Odours can interact with the olfactory receptors and elicit neuronal responses of the neurons.

B) Principle of combinatorial odour coding. The different types of olfactory receptors (here shown in four different colours) have different but overlapping receptive ranges for odours. When an odour ligand interacts with the respective olfactory receptor the olfactory receptor neuron gets electrically activated, which results in an influx of calcium (Ca$^{2+}$↑) into the neuron. Since, olfactory receptor neurons usually carry only one type of olfactory receptor in their membrane, each odour elicits a response pattern in a distinct set of olfactory receptor neurons (responsive neurons are marked by Ca$^{2+}$↑).
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In our group, we used the fruit fly *Drosophila melanogaster*. The fruit fly is a prominent model organism in several biological fields, including neurobiology. A large number of genetic tools have been developed over the years, some of which allow direct access to the physiology of the brain via calcium imaging. Neuronal activity can be visualized by a calcium-sensitive, fluorescent recombinant protein, GCaMP (Nakai et al., 2001; Tian et al., 2009). GCaMP is a sensor for the intracellular calcium concentration: when it binds calcium, it increases its fluorescence (see Fig. 2A).

When a receptor neuron responds to an odour, it is activated electrically and this leads to an influx of calcium into the cell. We then record a stronger fluorescence of GCaMP. Thus, the activation of neurons can be recorded under a microscope as a fluorescence increase.

When the olfactory receptor neurons express GCaMP, the fluorescent signals of these neurons can already be measured on the antenna of the fly, without the need of any preparation of the fly head (see Fig. 3A). Upon stimulation of the fly with an odour, a pattern of changed fluorescence on the antennal surface is visible and reflects the pattern of responsive receptor neurons. Thus, the fruit fly allows direct access to the physiological responses, without the detour via the animal’s behaviour.

But, when compared to dogs, is the fruit fly’s olfactory system as sensitive,
are the receptors equally broadly tuned, and is the odour-coding logic equally combinatorial? Indeed, the answer to all three questions is a clear yes! Regarding sensitivity, just think of how little time it takes to find a fruit fly on a wine bottle, once it was opened. Moreover, fruit fly’s olfactory receptor neurons also respond to substances, such as explosives, that are artificial and were irrelevant in the fly’s evolution (Marshall et al., 2010). Finally, odour coding in fruit flies is combinatorial: they express approximately fifty different types of olfactory receptors (Couto et al., 2005), which have different receptive ranges for odorants (de Bruyne et al., 2001; Hallem and Carlson, 2006). The combinatorial coding of odours by the fruit fly’s receptor repertoire has been shown in several studies and the results from all known olfactory receptor response profiles have been collected in a database (Galizia et al., 2010), as already reported in this Journal (Münch and Galizia, 2011).

The main difference, as compared to the dog, is that the fruit fly has "only" about fifty different receptor types (thus the theoretical capacity is limited to \(2^{50}\) patterns, still an astronomical number). However, there is also a major advantage: while in dogs the receptor neurons are located within the nose and are therefore inaccessible without invasive techniques, in fruit flies the receptor neurons are right beneath the outer surface of the antennae, directly accessible to microscopic imaging (see Fig. 3).

The fruit fly can serve as a natural receptor array for cancer

Thus, the next question would be whether cancer odours elicit different

Figure 3: Calcium imaging of the fruit fly’s antenna.
A) Experimental setup for calcium imaging of the fruit fly’s antenna. The schematic drawing (upper left) and the photograph (right) show the fixed fruit fly which is located under a fluorescence microscope. Odour stimulation is performed through a tube directed towards the antenna. Lower left: Picture of the antenna showing the basal fluorescence of GCaMP which is expressed in the olfactory receptor neurons.
B) The peripheral olfactory organs of the fly’s head comprise the antennae and the maxillary palps (only antennae were measured in the study). Olfactory receptor neurons stretch their dendrites into the antennae and are distributed almost randomly over the antennal surface. The responses of the fifty different receptor neuron types (here only three types are indicated by three different colours) to odours can be measured as distinct response patterns directly on the antennal surface. Note that this is a highly simplified representation. For example, receptor cells are also associated with different families of sensilla, which are hair-like structures that act as ‘sieves’ capturing odorant molecules from an air stream (schematic right).

Figure adapted from Strauch et al., 2014.
response patterns among the fifty different types of olfactory receptor neurons compared to healthy odours. We addressed this question in a recent study (Strauch et al., 2014). Using calcium imaging, we simultaneously recorded from approximately thirty receptor types on the antenna. Thus, we obtained a combinatorial pattern of activity across the antennal surface, and computer-analysed these patterns - just as a dog’s brain has to analyse the patterns of activity coming from its nose. In our study we investigated, 1) whether the fruit fly’s olfactory receptors respond to the odours emitted by cultured human cells, 2) whether the response to breast cancer and healthy cell lines differed, and 3) whether these differences are suitable to distinguish cancer from healthy cells.

VOC samples were obtained from the cell culture medium, in which the different cell lines had been cultured for 96 hours. We used the cell culture medium of five different laboratory breast cancer cell lines and one healthy control breast cell line, as well as a ‘pure’ medium control (without cultivated cells). After incubation, the cells were removed, so that only the medium was left. The medium was filled into headspace bottles and sealed, and a saturated VOC headspace above the medium was used for odour stimulation.

Calcium imaging is done on living animals, but any movement is detrimental to data acquisition. Hence, the flies and their antennae were fixed before they were positioned under a fluorescence microscope (see Fig. 3A). When we presented the different cell odours, we found strong responses in

![Image of calcium imaging results](image-url)

**Figure 4:** Fruit fly antennal responses to the stimulation with healthy and cancer cell odours.

A) The spatial response patterns differ for different cell odours. While no responses are visible to the N2 control (odourless), cell odours elicit strong responses, which can be measured as fluorescence changes ((F-F0)/F0). Responses are visualised using colours: strong calcium influx is shown in red (see false-colour bar to the right).

B) Selection of 300 informative response spots (pixels) on the antenna which were used for pattern analyses. Left: Image of the antenna expressing GCaMP. Right: Superimposed response spots. Each spot represents a place on the antenna and thus the response of an unknown number of olfactory receptor neurons. The response spots form spatially contiguous clusters with similar response dynamics (dots with the same colour belong to the same cluster).

C) Response time series of two response spot clusters. While the black cluster responds to most cell odours, the red cluster shows a different response profile, with particularly strong responses to the cancer cells (“canc1” to “canc5”). Each odour was presented twice (black bars at the top of the figure show the timing).

Figure adapted from Strauch et al., 2014.
the fly’s olfactory receptor neurons on
the antenna. The spatial response
patterns on the antenna were different
for the different VOC samples, but
reproducible, when the same odour
was presented twice. Next, we
analysed whether these patterns
contained information about the
health of the cells: we computed the
combinatorial pattern similarity
between odour responses. We selected
300 informative response spots (pixels)
on the antenna by unsupervised
feature selection. Each of the 300
selected response spots represented a
place on the antenna and thus the
response of an unknown number of
olfactory receptor neurons (see Fig.
4B). The 300 response spots could
further be clustered, when they
showed similar response dynamics.
These response spot clusters differed
in their responses to the odours: some
clusters responded to all odours, while
others showed odour-specific
responses). The response spot
positions were roughly conserved
between flies. The combinatorial
patterns allowed to discriminate the
cancer odours from the healthy control
odour and the medium control. This
discrimination could be visualised by a
principal component analysis (PCA)
which shows a projection of the
patterns in two dimensions (see Fig.
5). All cancer cell odours were in one
part of the plot, separated from the
healthy odour and medium control.
Additionally, we found a substructure
within the cancer cell odour cluster
with two distinct groups. A group of
two breast cancer cells lines evoked
dissimilar response patterns compared
to the other group of three breast
cancer cell lines (see Fig.5).

Figure 5: Representation of odours in a mathematical odour space (PCA-
analysis).

All cancer odours (“canc1” to “canc5”) are to the right and bottom in two
distinct clusters, while the control odours (“medium” and “healthy”) are clearly
separated at the top left.

Figure adapted from Strauch et al., 2014.
This revealed that cancer odours can indeed be distinguished from healthy odours by exploiting the combinatorial odour coding of the fruit fly’s olfactory receptor neurons. It should be noted that these experiments are a proof of principle, showing 1) that the olfactory receptors of the fruit fly can interact with VOCs derived from human cells, 2) that cancer and healthy cell odours elicit combinatorial response patterns of olfactory receptor neurons, and 3) that the odours from the healthy and cancer cell lines can be distinguished by these response patterns. As with many proofs of principle, the really interesting questions start now: are some receptors more important in the pattern than others, i.e. particularly responsive to VOCs of the healthy/cancer sample? Similarly, are some substances in the cell odours more predictive than others? And where does the difference between the different cancer cell lines come from? Most importantly: can we move this system from cell culture to real people?

What will cancer detection look like in the future?

So, will the future have flies flying around our heads, telling us about our health? Certainly not. This has a conceptual basis that is common in both, the fly system and the dog’s nose. It is this: ultimately, when analysing odour responses, we rely on memory of previous treatments. A dog has to be trained to particular samples, and the physiological response of a fruit fly needs to be calibrated. In dogs, the trainers have only limited access to sample material for training purposes, and need to use samples of the same patient several times. In a subsequent test situation, the dog can only generalise from the previously experienced cancer-specific odour components. In a particular study, the authors suggested that dogs may just learn to memorise a large number of different urine sample odours, rather than being able to extract the relevant cancer-specific odour information from the ‘trained’ urine samples (Elliker et al., 2014). A great advantage of the fruit fly is that training of the data analysis is not done with one individual animal, but across individuals. Thus, over time, a large database of cancer-related odour mixtures can be created, increasing the potential accuracy of the system.

Using body odours for cancer detection seems to be a promising approach for developing fast and non-invasive diagnostic tools, which might be particularly valuable when screening for cancer at early stages. Cancer detection by dogs demonstrates the high potential of natural olfactory systems for detecting cancer, because natural olfactory receptors are sensitive, fast in responding to odours, they have a large receptive range and, in their combination, they allow for discrimination of a tremendous variety of odours. However, as discussed earlier, the cancer detection by dogs is only indirect, because the human trainer has to interpret the dog’s behaviour. A more suitable approach is the direct access to the physiological response patterns, which underlie the odour discrimination. In our study, we could show that this direct readout of the response patterns is possible with the fruit flies olfactory receptors, directly on the antenna of the fly.

However, in the current state, the fruit fly approach is not at all fast, cheap or easy to handle, which would be the desired features of a diagnostic tool. Thus, intensive research is necessary to develop diagnostic tools which combine the desired features. A possible development of highly sensitive cancer detection tools could be to merge the advantages of the “fly detector” with the design of electronic noses. E-noses
are increasingly used in the medical context. Their current disadvantages are mostly a lack of sensitivity and a lack of knowledge to which chemical classes they need to/should respond. Thus, future research could focus on enhancing the sensitivity, the speed and the receptive range of e-nose sensors by not using artificial sensors in the array, but rather implementing natural olfactory receptors into e-nose arrays. The development of hybrid systems, combining the ease of use of e-noses and the high performance of natural olfactory receptors could then be a promising strategy for early cancer detection.
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