Receptomics: Tongue-on-a-chip with novel opportunities for food screening

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Abstract

Within the food and flavour industry it is considered a "holy grail" to measure the sensory aspects of taste and aroma quantitatively and independent from a taste panel. A microfluidic tongue- or nose-on-a-chip platform could be a system to emulate sensory perception into an instrument by monitoring the activation of specific taste and olfactory receptors in response to food samples. The complexity of food matrices, however, presents a large hurdle for the development and practical application of such systems. The issues are related to the frequent occurrence of (i) sample fluorescence, and (ii) non-specific host cell responses. In this paper we outline how we tackled these two issues with some practical examples; the sample fluorescence of coffee and the host cell response to bitter gourd and tomato juice. With the microfluidic receptor array developed in our lab, we were able to extract specific signals despite the sample fluorescence and host cell response effects. Reliable results could be obtained from these samples based on internal calibrations on-chip and sample controls. The fluidic measurement setup allows repeated exposures of the entire cell array to contrasting samples. With this strategy we could extract receptor-specific response trends to samples independent from generic host cell responses and sample fluorescence artefacts.

Keywords: taste receptors, biosensor, microfluidics, receptomics

Introduction

The term receptomics was first coined in 2004 to describe the systematic study of the receptorome, i.e. the collection of receptors and ligand-gated ion channels that control many physiological processes by serving as sensors for the cells and tissues that make up a specific organism [1, 2]. The class of G protein-coupled receptors (GPCRs) alone makes up an estimated 8% of the total human genome and represents an important target for food and drug research. The function(s) and ligand-specificity of many GPCRs of the human receptorome are still unknown [3, 4]. Additionally, the large number of single nucleotide polymorphisms (SNPs) and splicing variants of the receptor or ion-channel genes further increases their diversity for potential ligands, as well as the potential to couple to different G protein complexes [5-7]. As a consequence, the number of potential receptor conformations is remarkably high, and, therefore, a receptomics approach may promote the systematic screening of large compound libraries or complex natural food or health samples.

Receptomics requires an effective and efficient screening approach, but the conventional well-based, single use assays are notoriously costly especially for screening hundreds of receptors in parallel. Miniaturization and fluidics (sequential assays) potentially offer large advantages there. We used reverse transfection as method to make a live cell array on a carrier surface by simply printing a receptor DNA pattern on a slide and overlaying it with a cell line [8]. The expression of GPCRs in a cell array format was previously done by printing DNA arrays inside multi-well plates [9] or in fabricated micro reaction wells [10]. Combination with microfluidics, however, is considered the most promising road towards reducing the cost and increasing the throughput of screening receptor libraries [11].

The most fundamental difference between receptomics assays and end-point assays is the implementation of a microfluidic system and sequential exposures of the receptor cell array. While in an end-point assay the reagent and test mixture remain in the assay well, the receptomics cell array is exposed to the samples for a controlled period of time by the microfluidic system which sends a continuous flow of assay buffer across the cell array intermitted with injections of specific sample volumes from a sample loop [12, 13].

The microfluidic receptor cell array assay which was set up in our lab [14, 15] involves, in short, the expression of (taste) receptors in HEK293 cells co-expressing the $G\alpha16_{GUSTDUCIN44}$ chimera to target the signalling pathway towards intracellular calcium responses. Co-expression of the FRET-based calcium probe, Twitch2B [16], allows the live monitoring of changes in intracellular calcium levels resulting from specific receptor activations.

Taste receptors are located in specialized taste cells of the tongue and their specific activation in response to ingested food is interpreted by the brain. Bitter taste is recognized by the TAS2R receptor family for which 25 GPCRs have been identified in humans [17]. The ability to taste bitter substances is considered as an evolutionarily-developed mechanism that allows mammals to recognize toxins in plants. Since toxins are structurally very diverse, a wide range of bitter receptors have evolved to cover different classes of compounds. These bitter taste receptors have evolved into either broadly-tuned ones or more-specialized ones. Three TAS2R

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members (TAS2R10, -R14 and –R46) have a broad spectrum of agonists while TAS2R members R3, R5, R8, R13, R49, R50 and R9 are activated by only a few agonists known so far. While most bitter receptors have been de-orphanised, meaning that at least one agonist has been found, there are still a few orphan bitter receptors with unknown ligands, in particular TAS2R19, -R42, -R45, and –R60.

In this article we explore the potential of receptomics technology for analysing the bitter taste of coffee and bitter gourd and we explore the ability to identify bitter compound spikes in tomato juice. In whole food sample analysis we encounter two main bottlenecks: the effect of sample fluorescence in fluorescence measurements and the induction of a non-specific host cell calcium response after exposure to the food samples. In this article we show these effects, ways to compensate them, and we propose solutions to overcome the challenges to enable complex food receptomics studies.

Experimental

The cell culture conditions, receptomics setup, experimental condition, data analysis and statistical calculations were performed as previously described [14, 15, 18].

The coffee sample was prepared fresh on the day of measurement (45 grams of fine grounded coffee with 1L tap water), cooled to room temperature and diluted in assay buffer (NaCl 130 mM, KCl 5 mM, Glucose 10 mM, $CaCl_2$ 2 mM, HEPES 10 mM at pH 7.4). The bitter gourd fruit and tomato fruits were flash frozen in liquid nitrogen and grinded. The powder was defrosted and centrifuged to separate the insoluble fraction from the water fraction. The water fraction or juice was diluted in assay buffer. The tomato juice was spiked with Chloramphenicol 300 µM, D-Salicin 2 mM, Picrotoxinin 300 µM, Denatonium Benzoate 300 µM, Aristolochic acid 30 nM and PROP 10 µM.

Results and discussion

Sample fluorescence of food samples

When samples are fluorescent to the extent that it interferes with a fluorescence-based receptor cell assay one solution is to dilute the sample until the sample fluorescence is low enough, although this will also dilute the sample's bioactivity. We tested this approach using coffee as a pilot experiment. Coffee contains alkaloids which contribute to a high level of fluorescence [19], that is detected by the CFP and YFP emission filters (Em 480/40 and 535/30 respectively) of the FRET based calcium sensor Twitch2B. We used 8x diluted coffee in assay buffer as optimum to balance the high levels of sample fluorescence while maintaining detectable bitterness in the sample.

An array of bitter taste receptors was exposed to a series of injections of 8x diluted Arabica and Robusta (light roast, AL and RL respectively) coffee. A set of 23 bitter taste receptors was assayed including 2 polymorphisms for R4 and R46 together with a mock control. To monitor any effects on the fluorescence caused by the sample we included a fluorescence control (YC-). This is a modified version of the calcium sensor where a deletion in the calmodulin and M13 domain makes the protein inactive for sensing calcium. This allows the monitoring of any sample effects on the baseline fluorescence. Samples that display fluorescence at CFP excitation wavelengths usually give increasing signals in both the CFP and YFP emission channels.

To separate sample fluorescence from the ratiometric FRET signal, our current solution is a computational method to separate background from actual signal. Using the ratiometric properties of the probe it is possible to separate the sample fluorescence –a signal that increases in both CFP and YFP channels- from a signal that originates from the ratiometric calcium probe - a FRET signal that decreases in the CFP channel and increases in the YFP channel. This method is still under development, but could efficiently eliminate sample fluorescence from a fluorescein control and from 8x diluted coffee. Figure 1 shows the raw FRET ratio signals and sample fluorescence or background (BG) corrected signals of a measurement series for 11 sample injections. The charts show signal curves for the controls Mock and YC- and one of the 23 bitter taste receptors; TAS2R16. The injection series is measured with an injection interval of 5 minutes and an exposure time to the samples of 30 seconds. The fluorescein injection serves as a control injection for auto-fluorescence background. At a concentration of 1 µM the fluorescein dye results in a fluorescence signal in both emission channels, but it does not elicit a cellular calcium response. The ATP injection is a control which triggers a host cell calcium response [20] and the D-salicin injection is a control bitter compound for TAS2R16 ensuring a response from transiently expressed bitter receptor on the cell array.

After the computational separation of signal and background the resulting signal remains for ATP and the D-salicin sample for TAS2R16. The YC- control line is clean of the sample fluorescent background signal. The coffee sample still shows fluctuations in intracellular calcium which indicates that even at 8x dilution the coffee still triggers a non-specific host cell calcium response in the HEK293 cells.

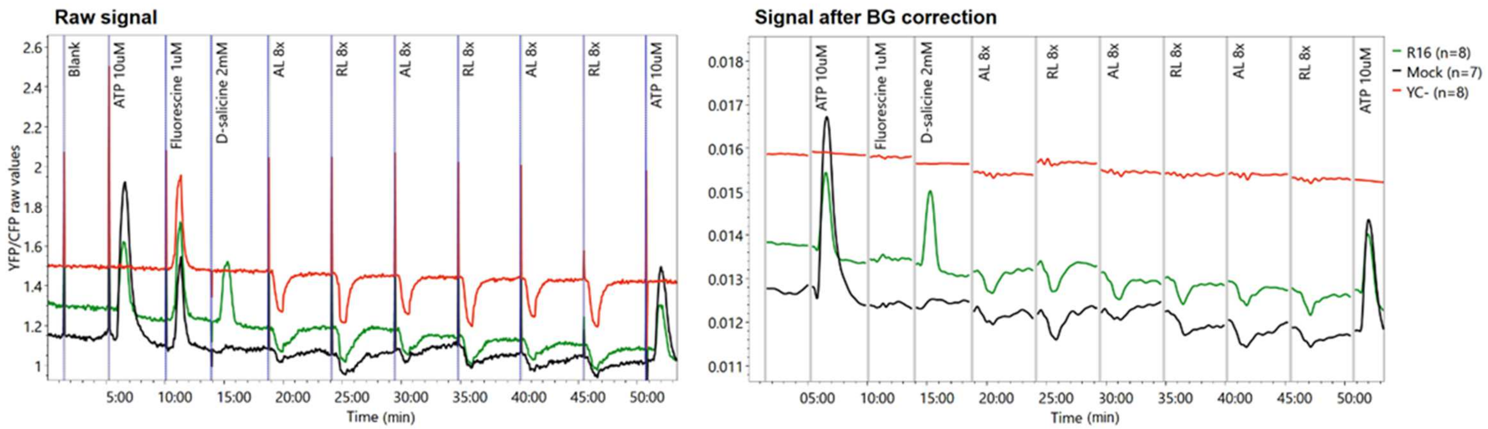

Figure 1*: Raw YFP/CFP ratio curves (left) and background (BG, right) corrected curves for an injection series of coffee samples Arabica and Robusta light roast (AL and RL) 8x diluted in assay buffer. Curves are showing for controls Mock and YC- and TAS2R16 bitter taste receptor. In brackets are the number of spots on the cell array.*

To detect specific signals for any of the bitter receptors we analysed the signals of all 23 bitter taste receptors coded on the cell array. The Arabica and Robusta light roasted (AL and RL) samples were injected sequentially in alternating order and in three replications. With several alternating and repeated injections, a spot-based response trend can be determined showing inter-sample differences based on a linear mixed model [18]. This spot-based data analysis considers each spot as a separate measurement. Because each spot is serially exposed, this allows us to draw conclusions about differences between samples and eliminate the variability between spots. In this way we can separate the considerable biological variability between spots of the same type from the relevant information, that is, the variation in responses between the two samples. This statistical analysis is shown in Figure 2. Bitter taste receptor TAS2R8 is the only receptor displaying a clear specific response to the Robusta coffee sample relative to the blank injection. However, focusing statistically on inter sample differences, comparing AL to RL, the contrast shows a higher bitter receptor activation in Robusta coffee for TAS2R8, R14 and R50.

Figure 2*: Statistical comparison of Arabica and Robusta light roast (AL and RL) 8x diluted in assay buffer to the blank (top) and an inter-sample difference (bottom). In both analysis the responses are normalized to the Mock.*

In conclusion, although the signals in the analysis of 8x diluted coffee were low, some sample differences based on bitter receptor activations were detectable. However, overall, the bitter response fingerprint may not be complete due to the dilution. The fluorescence-based detection method, even with smartly designed computational methods, may not be very suitable for the measurement of undiluted samples like coffee because the dynamic range for measuring receptor activation becomes comparatively smaller resulting in noisier signals. A way to counter the problem of sample fluorescence may be to switch to the use of bioluminescent probes and detection systems that do not depend on external excitation light. Suitable sensors would be the bioluminescence calcium sensor GeNL(Ca^{2+}) 60-520 based on split NanoLuc [21] or the use of a BRET based calcium sensor CalfluxVTN [22].

Host cell responses of complex samples

One advantage of the microfluidic receptomics platform is that it enables sequential injection of samples and determination of differences between samples. But when these samples are complex mixtures, extracts or even food products, there can be non-specific host cell calcium responses triggered in the cell that bear no relation to the specific receptor activations of interest. These endogenous host cell responses may be caused by pH or salt perturbations in the samples, but usually these parameters can be minimized by adjusting the conditions of the assay. However, samples may also contain compounds that activate a host cell receptor or contain toxic compounds, and then the assay will measure a positive or negative host cell response in the mock transfected spots (spots without recombinant receptor). One straightforward solution is to dilute the sample until the host cell response is below threshold. This solution will not be applicable to all types of samples, but if bioactivity is stronger than the host cell response a sample dilution may help to separate specific responses from host cell responses. This was shown earlier for a crude chilli pepper extract that was measured for bioactivity with the TRPV1 ion channel [13]. At a 300-fold dilution of the extracts, there was no generic host cell response to the extract anymore, but there was still a clearly specific TRPV1 ion channel activation.

Similarly, here we show results with bitter gourd juice. This bitter gourd sample was diluted 30-180-fold in assay buffer and exposed to the array of bitter taste receptors, see Figure 3. The sample did not display sample fluorescence, but there was a considerable concentration dependent nonspecific host cell response in the Mock spots. Superimposed on this host cell response, however, the spots coding bitter taste receptors TAS2R16 and TAS2R8 show additional strong calcium activation peaks that become more distinct from the host cell response at the higher dilutions. The TAS2R14 shown in Figure 3 along with the other 21 bitter taste receptors on the array did not show a specific response. Thus by analysing this crude vegetable extract, we can pinpoint specific bioactivity to TAS2R16 and TAS2R8. Follow-up studies may be conducted to identify the metabolites responsible for this strong bitter taste.

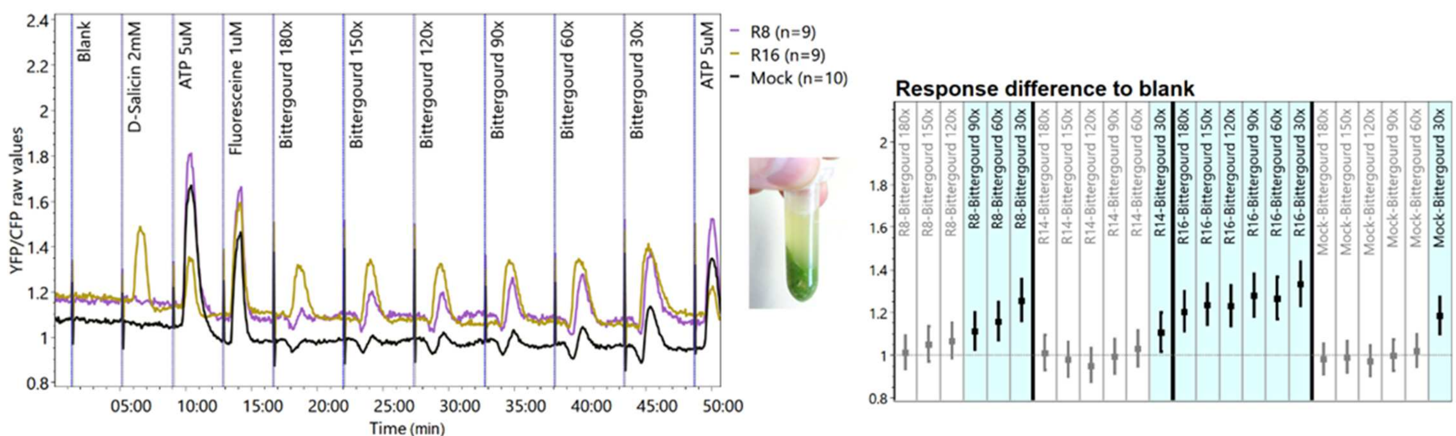

Figure 3*: Raw YFP/CFP ratio curves (left) and statistical comparison (right) of bitter gourd fruit juice diluted in assay buffer. Showing responses for TAS2R8, TAS2R16 and Mock and including TAS2R14 as reference in the statistical analysis.*

Unfortunately, it is not always possible to sufficiently dilute a sample to keep the host cell response within acceptable limits, without losing the bioactivity as well. However, we found that with the aid of a simulated host cell response –a host cell response to ATP- the response can be normalized in a spot-dependent manner using the ATP injections as a reference in a two sample contrast [18]. Bitter receptor spikes were retrieved from the sample comparison even though the host cell response was considerable and variable between spot types. This approach was demonstrated using tomato juice samples spiked with bitter compounds as shown in Figure 4 (ECRO 2018

conference poster [23]). This result showed that a receptor-specific calcium response may be cumulative to a host cell response. In a direct contrast of 5-fold diluted tomato juice with and without bitter compound spikes and using the ATP response to normalize the responses, 6 out of 8 bitter spikes were recovered. However, the approach of using an ATP host cell response correction only works when it accurately mimics the host cell response. This can be determined in a response correlation between the ATP responses and sample host cell responses. Other sources of intracellular calcium modulation, like the activation of ion-channels for example, may potentially introduce artefacts.

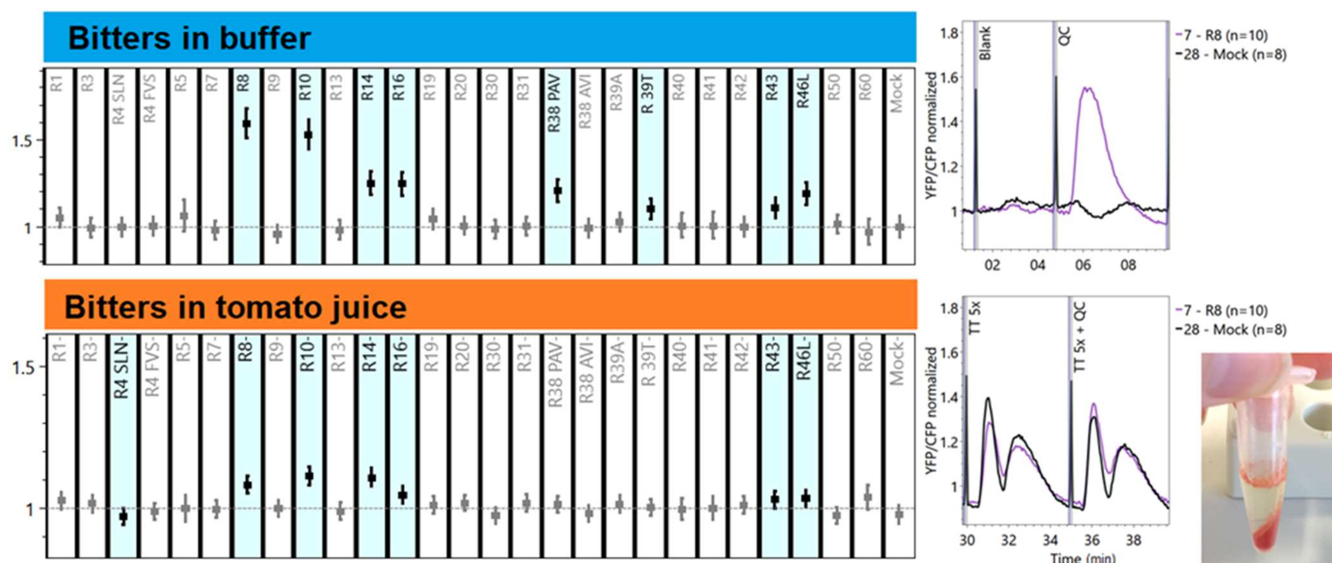

Figure 4: *Statistical comparison of bitter compounds in buffer (top) and a comparison of tomato juice diluted 5x in assay buffer with and without bitter compounds (bottom). Bitter compounds Chloramphenicol 300 µM, Denatonium Benzoate 300 µM, Picrotoxinin 300 µM, D-Salicin 2 mM, PROP 10 µM and Aristolochic acid 30 nM were used which activate TAS2R8, R10, R14, R16, R38PAV and R43 respectively. TAS2R46 is activated by several compounds. The response of TAS2R8 to Chloramphenicol in buffer (QC) and in 5x diluted tomato juice (TT) is displayed in the small graphs on the right.*

As long as a calcium read out system is used to detect receptor activation of complex samples, the host cell response remains something that must always be considered. There is a need, therefore, for a different read-out system that is receptor-specific and not based on second messengers like calcium or cAMP. An example is the miniG system introduced by Wan in 2018 [24]. Both the receptor and G protein are fused to parts (Smbit and Lgbit) of a split nanoluc enzyme. Only when the receptor-Smbit is activated and the Lgbit-miniG protein binds the receptor the luciferase is reconstituted and starts to emit light. This system promises to offer an elegant way of eliminating host cell response interference by literally keeping those responses in the dark. Pilot results show that this system is applicable and detectable in a cell array format as well.

Conclusion

For taste and flavour research the most promising feature of the receptomics platform is the possibility of screening and comparing complex samples (matrices). In product development or crop breeding, subtle sample differences in taste are difficult to measure using taste panels where subjectivity can be an issue. If this receptomics platform could provide an efficient means of pre-screening samples/varieties this could become a valuable tool. Preliminary results have indicated that indeed the receptomics platform is able to measure sample differences in vegetable extracts and beverages despite the complexity of the samples if appropriate dilutions are chosen (sample fluorescence and host cell responses). Bioluminescent assays promise to bridge the step to undiluted samples.

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