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Nanowire biosensors with olfactory proteins: towards a genuine electronic nose with single molecule sensitivity and high selectivity

Sangjun Noh[1], **Francesco Tombola**[2] **and Peter Burke**[1]

[1] EECS, UC Irvine, Irvine, CA, United States of America
[2] Dept. of Physiology and Biophysics, UC Irvine, Irvine, CA, United States of America

E-mail: pburke@uci.edu

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Abstract

We describe the concept and roadmap of an engineered electronic nose with specificity towards analytes that differ by as little as one carbon atom, and sensitivity of being able to electrically register a single molecule of analyte. The analyte could be anything that natural noses can detect, e.g. trinitrotoluene (TNT), cocaine, aromatics, volatile organic compounds etc. The strategy envisioned is to genetically engineer a fused olfactory odorant receptor (odorant receptor (OR), a membrane-bound G-protein coupled receptor (GPCR) with high selectivity) to an ion channel protein, which opens in response to binding of the ligand to the OR. The lipid bilayer supporting the fused sensing protein would be intimately attached to a nanowire or nanotube network (either via a covalent tether or a non-covalent physisorption process), which would electrically detect the opening of the ion channel, and hence the binding of a single ligand to a single OR protein domain. Three man-made technological advances: (1) fused GPCR to ion channel protein, (2) nanowire sensing of single ion channel activity, and (3) lipid bilayer to nanotube/nanowire tethering chemistry and on natural technology (sensitivity and selectivity of OR domains to specific analytes) each have been demonstrated and/or studied independently. The combination of these three technological advances and the result of millions of years of evolution of OR proteins would enable the goal of single molecule sensing with specificity towards analytes that differ by as little as one carbon atom. This is both a review of the past and a vision of the future.

Keywords: electric nose, lipid bilayer, olfactory odorant receptor (OR), nanowire, nanotube

(Some figures may appear in colour only in the online journal)

1. Introduction

Mark Reed's lab at Yale University developed extensive silicon nanowire biosensor technology over his vast and illustruous career. His lab demonstrated pH sensing with 0.01 pH resolution [1], label-free detection of enzyme−substrate interactions [2], label-free biomarker detection from whole blood [2, 3], regenerative electronic biosensors [4], and top-down massively parallel fabrication of biochips compatible with conventional CMOS foundries and fabrication steps [5]. This led to unprecedented sensitiviy of analytes, down to attomole, and specificity at the level of antibody discrimination.

In this work, we describe a general framework and paradigm as well as a specific, detailed 'roadmap' of how the next revolution of nanowire biosensors can and will extend this work to the ultimlate limit of single molecule sensitivy

and selectivity among analytes that differ by as much as one carbon atom. This is both a review of the past and a vision of the future of this field. The overall strategy is to build on billions of years of evolution of olfactory proteins and implement this in an on chip electronic sensing format. The technological building blocks have been demonsrated by the author's group, in collaboration with the Mark Reed Lab, and other groups around the world. The way these pieces fit together into an *integrated nanosystem* will be described in detail in this paper.

Although we focus on silicon nanowires here (with the advantage of top-down fabrication pioneerd by Mark Reed's lab), this general approach to functionalize electronic biosensors can be used for any type of electronic sensor, for example graphene and other Van Der Waals 2D materials, carbon nanotubes, bottom up Si nanowires, III–V and II–VI bottom up nanowires such as GaAs, ZnO, InP, and even tertiary compound bottom up nanowires. Therefore, in this paper, we use the term nanotube and nanowire as a generic term for nanosensor.

The anticipated outcome and end goal is the ability to sense anything the nose can, from TNT to polycyclic aromatic hydrocarbons, etc. For example, anything that can be detected by sniffing dogs could be detected if the olfactory receptors and ligands were known. This lays the scientific foundation for chemical weapons detection systems with low false positives, as well as improvised explosive devices (IED) detection. In addition, this technique lays the groundwork for developing high precision chemical detection in general which can be utilized in a variety of applications requiring high precision process control (i.e. advanced manufacturing). Finally, since this sensor is electronic it could be integrated in the future into a massively parallel e-nose system (read out with a simple wireless interface with a cell phone) with signal recognition based on hundreds of different odorant receptor (OR) sensors integrated onto one chip: a genuine electronic nose.

2. Olfactory proteins: accomplishments to date

2.1. Olfactory proteins: mechanism of action

Figure 1 (from [6–8]) shows the mechanism of action of natural (wild-type) olfactory proteins upon binding of an odorant to an OR membrane protein: the OR changes its conformation, resulting in change in cytosolic [cAMP]. Like other G-protein coupled receptors (GPCRs), OR proteins change their conformation slightly upon ligand binding on the extracellular side of the plasma membrane (cell surface). In biological systems, this subtle conformational change causes cytosolic trimeric G proteins (GPTases) in the cytoplasm to become activated. In olfactory receptor neurons, this leads to a cytosolic cascade, which causes the opening of both cAMP-gated Na^+ channels and Ca^{2+}-activated Cl^- channels, ultimately triggering the action potential of the neurons and sending a signal to the brain. The OR proteins are the result of millions of years of evolution for sensing food sources,

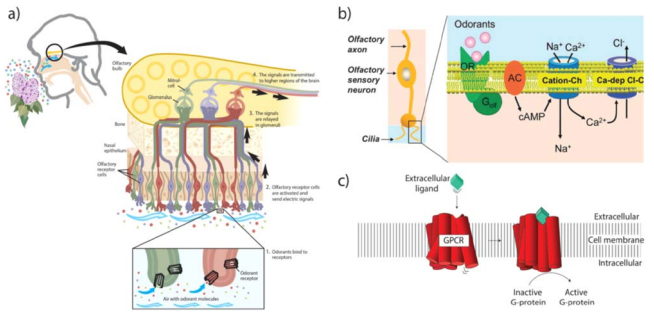

Figure 1. (a) Overview of the mammalian olfactory system. Reproduced with permission from [6]; (b) mechanism of action of OR proteins: the binding of an odorant causes a change in cytosolic [cAMP], which in turn causes ion channels to open and neurons to fire. Reproduced from [7] with permission from the Royal Society of Chemistry; (c) schematic cartoon of subtle conformational shift that occurs during OR-ligand binding. Reproduced from [8], with permission from Springer Nature.

danger and reproductive/social signals, of a variety of organisms [9]. Approximately 1000 different OR genes are present in the mammalian (including human) genomes [10], consisting of 3% of all human genes. When counting intact (functional) OR genes, humans have approximately 400 while elephants have 2000 [11]. A review of all odorants is beyond the scope of review [11], and in fact not all odorants and ORs have been characterized. The 2004 Nobel Prize in Physiology was awarded to Linda Buck and Richard Axel for their discovery of this class of genes [10].

The evolutionary advantages of these proteins are that they can be sensitive to specific chemicals. OR receptors can be precise (e.g. those detecting pheromones), but transduction to ion channels is via change in cytosolic [cAMP]. Replicating this precise pathway, with soluble proteins and enzymes, is something that is hard to engineer into an electronic-nose (e-nose), since it would be preferred to minimize the number of components that need stabilization. We discuss next initial attempts in this direction that have shown promising results and point to new approaches that simplify this elegant biological mechanism.

2.2. Olfactory protein coupling to nanotubes

Two groups have managed to tether lipid bilayer bound ORs directly to nanotube electronic devices (Hong *et al* [12], Johnson *et al* [13]). When the OR binds to a ligand, the conformation change of the OR, even though extremely slight (less than an Angstrom shift in the location of the alpha helices), causes a change in the nanotube conductance. This is possible because nanotubes are extremely sensitive to nearby charges, able to sense on the order of a single electron of equivalent charge even in solution at room temperature [14]. Amazingly, this engineering of the OR to the nanotube network creates a system that is selective for two different analytes that differ only by a single carbon atom (figure 2 from [12, 13]). This tethering of OR directly to nanotubes achieves *unprecedented selectivity,* but *limited sensitivity* (i.e. *not single molecule as we envision in this paper*). The selectivity comes from the evolved OR recognition motifs, but the

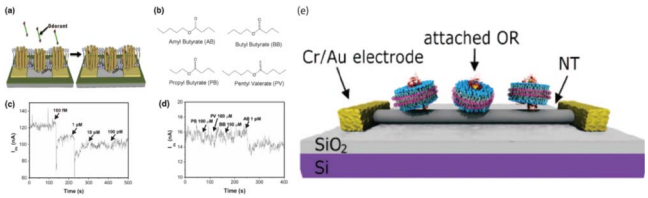

Figure 2. (a)–(d): Sensing different analytes with specificity differentiating between molecules that only differ by a single carbon atom. [12] John Wiley & Sons. Copyright © 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim; (e) an alternative method developed by Penn to attach ORs to nanotubes with lipid bilayer disks. Reprinted with permission from [13]. Copyright (2011) American Chemical Society.

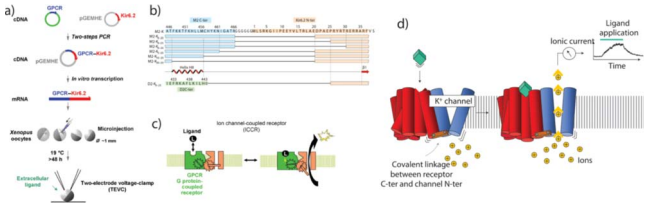

Figure 3. (a), (b) Genetic splicing technique (common tool in molecular biology labs) which fuses the GPCR (i.e. OR) and ion channel (Kir6.2) into a single mRNA strand cellular injection followed by protein synthesis of the fusion protein coded by the mRNA strand. Reprinted from [15], Copyright (2015), with permission from Elsevier; (c), (d) cartoon of membrane bound fusion protein and it's electrical activity of the blue (ion channel) domain upon binding of the OR domain (red) to a ligand. Reproduced from [8], with permission from Springer Nature.

extremely subtle change in OR protein shape on binding is difficult to register electrically with the carbon nanotube.

2.3. Olfactory protein coupling to ion channels

The disadvantageous aspect of OR receptors for integration with nanoelectronics is that they only change their conformation ever so slightly upon binding to a ligand. This results in a weak readout signal. In the cytosol in cells, the [cAMP] concentration change causes ion channels to open leading to membrane depolarization and firing of electrical signals. This is the built-in amplification mechanism in living systems that enables small odorant concentrations to be transduced into the firing of neurons. Recently [8], Moreau *et al* was able to engineer a hybrid GPCR-ion channel system, which covalently tethered the receptor to an ion channel, simply by genetically splicing the two proteins together and allowing them to be expressed naturally in a host (Xenopus oocytes). Figure 3 (from [8, 15]) illustrates the mechanisms through which binding of an OR to a ligand can open an ion channel in suspended lipid bilayers. This mechanism (as demonstrated in the experiments [8]) bypasses the need for a [cAMP] change in cytosol and concentrates the entire molecular sensing apparatus into one membrane-bound protein.

Moreau *et al* originally fused GCPR to a K+ ion channel in 2008 [8]. The detailed methods were published in Methods

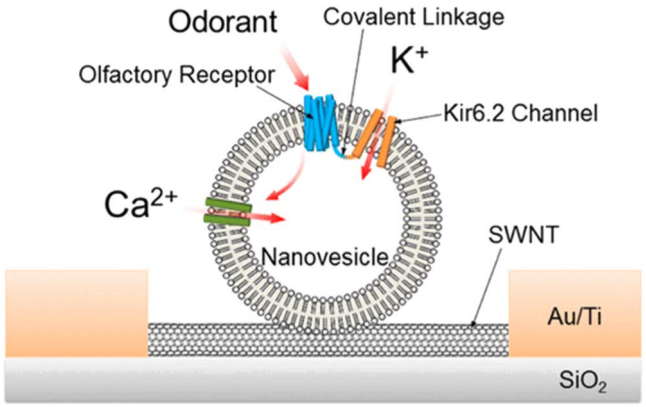

Figure 4. Lysosome on nanotube with ion channel/GPCR integration. Reprinted with permission from [22]. Copyright (2015) American Chemical Society.

in Enzymology in 2015 [15]. In 2012 they coupled it to rhodopsins showing successful light to ion channel conversion (albeit at the ensemble, not single ion channel, level) [16]. Coupling to the β_2-adrenergic receptor class of GPCRs (involved in motion and smooth muscle, hence a target of β-blocker class of drugs) was presented in 2011 [17]. Incorporation with the human oxytocin receptor was published in 2014 [18]. Recently [8] Moreau *et al* showed that the ion channel to GPCR linker chemistry can make the ligand induce or repress ion channel opening by design [19]. A similar fusion protein (ion channel + OR) was demonstrated by Hong *et al* with integrated florescence readout [20]. Electronic readout with nano-transistors is discussed next.

2.4. Integration of OR coupled ion channels into vesicles on nanowires

In order to exploit the advantages of the Moreau *et al* approach [8] (which combine the relative large ion channel currents with the OR (GPCR) selectivity), Hong *et al* [12] have recently incorporated these hybrid OR-ion channel membrane protein sensors into lipid vesicles. Prior art with lipid vesicles containing OR provided limited success [21]. Incorporation of the Moreau *et al* [8] ion-channel OR hybrid into the vesicle improved the results. The scheme is shown in figure 4 (from [22]). The vesicles containing the membrane proteins are dropped onto a nanotube where they are immobilized via physisorption. Upon binding to an odorant, an ion channel opens, charging the vesicle up, which the nanotube detects electrically [22]. This approach is simple and elegant, but still does not take full advantage of the ion channel currents: the ion channel currents never directly interact with the carbon nanotube. In our work we have directly incorporated ion channels onto nanotubes as discussed next.

2.5. Ion channel coupling to nanowires

The author's lab has shown the ability to interrogate single ion channels using lipid bilayers tethered either covalently

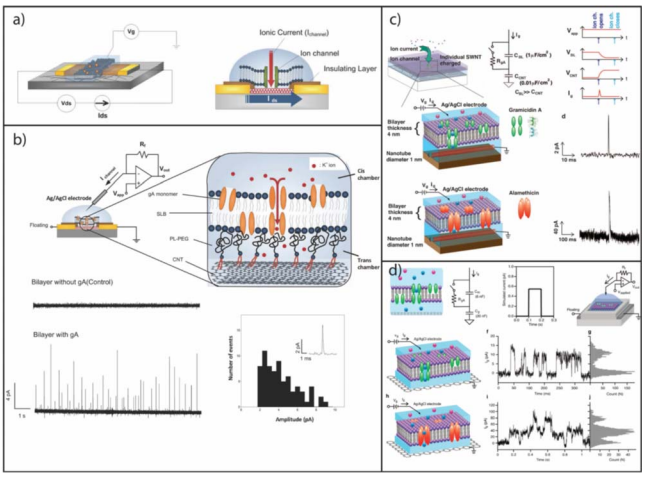

Figure 5. (a)–(c) Nanotube network and functionalization with polymer cushion, with clear demonstration of sensing of the opening and closing with single ions. Reproduced from [23]. CC BY 4.0; (d) graphene sensing of single ion channels including alamethicin and gramicidin. Reproduced with permission from [24].

or non-covalently to a variety of nano-electronic devices, including carbon nanotubes [23], graphene [24], and silicon nanowires [25]. We have done this for gramicidin and alamethicin, both textbook ion channel proteins. Figure 5 (from [23, 24]) shows a summary of our results from this platform.

2.6. Tethering chemistry of lipid bilayers to nanotubes/ nanowires

There are a variety of covalent and non-covalent techniques to attach a lipid bilayer to a solid surface. These have been heavily researched over the last 10–15 years [26–29]. Physisorption to a hydrophilic surface such as glass is the simplest technique; adding a hydrated 'polymer cushion' layer between the lipid bilayer and the solid surface enables better studies of membrane bound proteins with either electrophysiological or mechanical properties that depend on access to a hydration layer on both sides of the bilayer, in particular between the lipid bilayer and the solid surface.

Recently, in the last few years, the author's lab has applied a large variety of these tethering techniques to nanotube, graphene, and nanowire device platforms fabricated in house (nanotube, graphene) and at our collaborator M. Reed's lab at Yale (silicon nanowire). We have successfully demonstrated that these tethering chemistries (covalent silanol based chemistry [30], physisorption onto a poly-L-lysine cushion [23], and physisorption onto PEG polymer cushion [23]) sustains ion channel activity in the lipid bilayer membrane, which can be detected by the integrated nanowire systems. This provides strong confidence that these tethering chemistries, heavily studied inthe author's lab, are compatible with the envisioned OR-ion channel fusion protein system developed independently by Moreau *et al* [8] for suspended lipid bilayers.

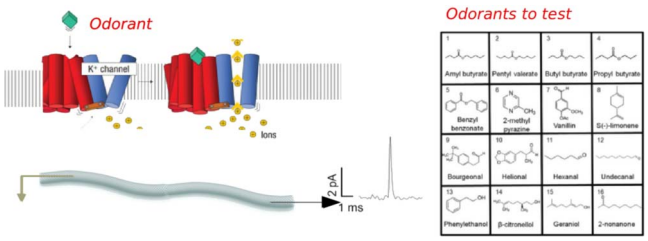

Figure 6. Envisioned approach: couple a nanotube (or nanowire) to a fused ion channel/OR membrane protein. When a ligand odorant binds to the OR, the ion channel fires. The nanotube (or nanowire) amplifies the ion channel current, and the amplified current is read out with an external circuit. Reprinted from [20], Copyright (2015), with permission from Elsevier.

3. Towards an olfactory protein-based e-nose

3.1. Overview

Our proposed approach takes the next logical step, and incorporates the ion channel-OR hybrid into a lipid bilayer that is directly on top of the nanotube or nanowire. In this way, when the ion channel fires, it *directly* interacts electrically with the nanotube or nanowire, charging it and changing its conductance *dramatically*. Quantifying how dramatically will be an important next step, but estimates are that a few pA of ion channel current incident on the sidewalls of the nanotube or nanowire can switch a nanotube or nanowire completely from the on state, carrying μA of current through the wire, to off state (carrying zero current), hence achieving an amplification factor of *one million*. Because the OR is integrated, the *selectivity is preserved*. Based on this concept, if a *single* odorant molecule binds to a single OR receptor causing the ion channel to fire, we should see a change of about one μA of current through the carbon nanotube or nanowire, *a current gain of* 1 *million.*

A very promising approach is shown in figure 6 below (odorants from [20]). It has the following potential advantages:

- Exquisite selectivity based on millions of years of evolution
- Single molecule sensitivity
- Single ion channel readout (potential high gain system)

We have broken the project into three important steps:

3.2. Step 1: preparation of fusion constructs and functional tests

3.2.1. Step 1A: fusion of OR to potassium channel and protein expression. Moreau *et al* 2008 [8] constructed different fusion proteins connecting the M2 muscarinic acetylcholine receptor to the inward-rectifier potassium channel Kir6.2. Based on our previous experience in designing and generating chimeric [31] and concatenated [32] ion channels, we envision the custom synthesis of complementary DNAs (cDNAs) coding for the fusion protein OR-K_{full} (figure 7) [33, 34] in which the C-terminus of the olfactory receptor hOR2AG1 is connected to the N-terminus of mouse Kir6.2 by

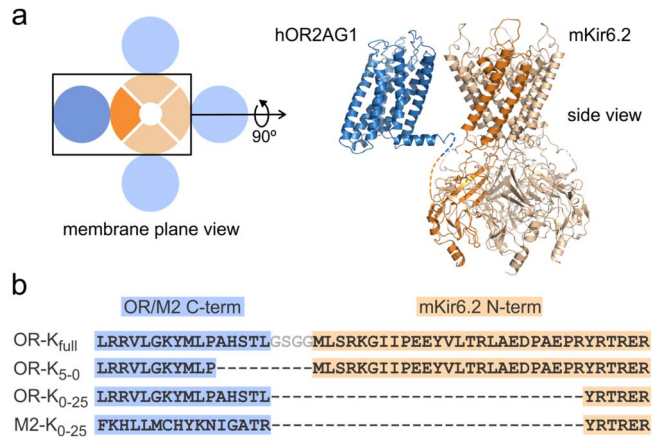

Figure 7. (a) Design of OR-K fusion proteins. The channel component is in orange, the receptor component is in blue. One of the four subunits is shown in darker shade. The structure for mKir6.2 is from Li *et al* 2019. Homology model for hOR2AG1 was generated using I-TASSER based on templates identified by LOMETS. (b) Examples of linkages between OR and channel components that will be examined compared with the most effective linkage between the M2 receptor and the same channel from Moreau *et al* 2008.

the linker GGSG. The constructs can be subcloned in a plasmid for protein expression in Xenopus oocytes such as pGEMHE. The cDNA coding for the control fusion protein M2-$K_{0–25}$ (from [8]) can be also custom synthesized and similarly subcloned (figure 7). cDNAs with different deletions within the OR—Kir6.2 connecting region (e.g. OR-$K_{0–25}$, (figure 7) can be generated from the OR-K_{full} construct using standard PCR techniques [31]. Constructs in which the OR and Kir6.2 are coded as separate proteins (negative controls) can be produced using the same techniques. Plasmid DNA containing the various constructs are then used for *in vitro* mRNA synthesis. mRNAs are injected in Xenopus oocytes for functional studies using electrophysiological methods (next section). After functional characterization of the fusion proteins, the OR-K construct displaying the largest response to the ligand can be subcloned in a vector suitable for expression in mammalian cells. Retroviral vectors can be used to produce a cell line stably expressing the construct of interest under a CMV promoter and a selectable marker such as puromycin. A Hemagglutinin (HA) tag can be added at the C-terminus of the construct to validate protein expression in the cell line by immunocytochemistry (ICC). When expressed in heterologous systems, olfactory receptors often fail to reach the plasma membrane, due to the lack of odorant receptor-specific accessory proteins that are required for proper trafficking [35, 36]. hOR2AG1 was previously shown to be targeted to the plasma membrane of heterologous cells when co-expressed with the heat shock protein Hsc70t [37]. Hence, Hsc70t can be co-expressed with the isolated OR and with the OR-K fusion constructs either by mRNA co-injections in Xenopus oocytes, or by adding an internal ribosome entry site sequence followed by the Hsc70t cDNA to the 3' region of the constructs for expression in mammalian cells. Plasma membrane targeting of the fusion proteins can be assessed by confocal microscopy in colocalization experiments with a plasma membrane marker.

3.2.2. Step 1B: electrophysiological measurements of construct activity. The function of M2-K fusion proteins was tested via electrophysiological measurements by Moreau *et al* 2008 [8]. The proteins were expressed in *Xenopus* oocytes and the response to ligands (acetylcholine and the muscarinic agonist carbachol) measured in two-electrode voltage clamp (TEVC). Based on our experience in the functional characterization of ion channels [38, 39], and the use of ligands to modulate ion channel activity [40, 41], we envision to express the OR-K constructs in the same system as Moreau *et al* [8] and use of TEVC to measure the response to various concentrations of amyl butyrate, the reported ligand for hOR2AG1 [37]. The response can be quantified as % change in potassium current induced by the ligand. As a negative control, one can utilize a condition in which the OR and the potassium channel are expressed as separate proteins [8]. The positive control can be based on the response of M2-$K_{0–25}$ to acetylcholine measured under the same conditions. The OR-K construct with the strongest response can be selected for further use.

3.2.3. Expected outcomes and alternative approaches to step 1: If step 1 as outlined above fails, there are alternatives. With the strategy outlined above, one expects to identify a fusion construct that will display responses to the odorant ligand comparable, in magnitude, to the response of M2-$K_{0–25}$ to acetylcholine. The co-expression of Hsc70t is expected to be sufficient for proper membrane trafficking of the isolated OR [37]. However, linking the OR to the potassium channel may result in a suboptimal plasma membrane localization of the fusion protein even in the presence of Hsc70t. In this case one can try co-expression of other accessory proteins that have been previously shown to allow effective OR trafficking in heterologous cells. For example, one can use the short version of receptor-transporting protein 1 (RTP1S), and the olfactory guanine nucleotide exchange factor Ric8b following the protocol from Zhuang and Matsunami, 2008 [42].

3.3. Step 2: couple fused protein in lipid bilayer to nanotube/ nanowire and demonstrate specificity

The goal here is to tether the lipid bilayer system to the nano-electronic system. A demonstration/ proof of concept can be applied to any arbitrary nanowire type system, be it graphene nanoribbons, silicon nanowires, individual single walled carbon nantoubes, III–V nanowires (even optically active ones), and any other nano-transistor from the nano-wire genre. In fact, we have recently published a method [30] to tether lipid bilayers to a large class of nano-electronic devices, shown in figure 8 (from [30]). We demonstrated many of these platforms as single ion channel detectors [23, 24].

For example, silicon nanowire devices can be made massively parallel using only optical lithography.

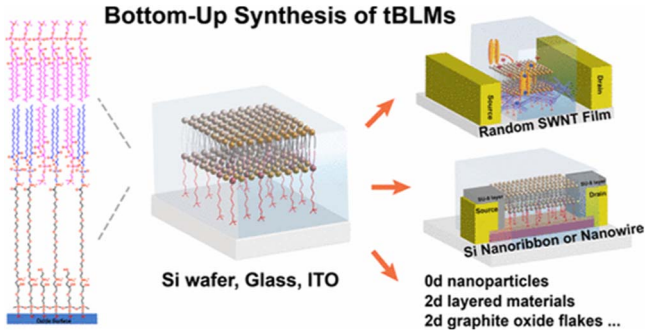

Figure 8. Burke Lab recently developed a versatile lipid bilayer functionalization scheme that can be applied to a variety of nano-electronics platforms. In this work, we will pick one platform for demonstration and proof of concept purposes. Reprinted with permission from [30]. Copyright (2017) American Chemical Society.

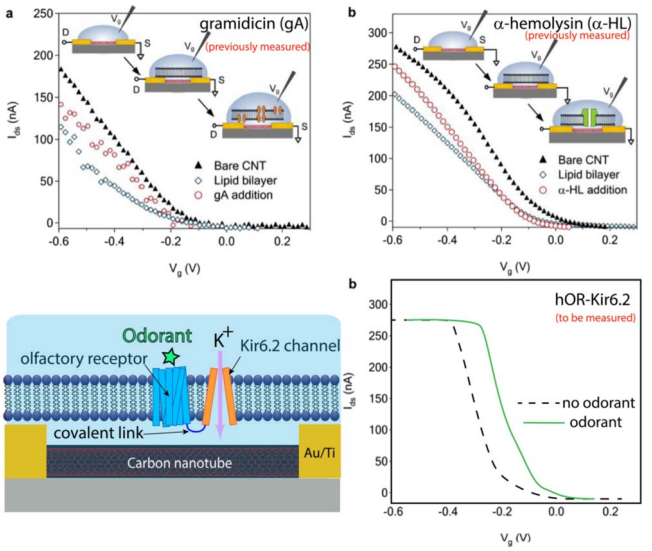

Figure 9. (Top) Prior measurements of depletion curves in the presence and absence of ion channels. (Bottom) Envisioned measurements and schematic of system.

For step 2, the scientific goal would be to demonstrate sensing of one analyte over the other (i.e. specificity), and to look at the DC (time average) current as a result of large numbers of ion channels. This was already demonstrated with OR alone (not ion channels coupled) by both Hone *et al* and Johnson *et al* so one has high confidence it can be demonstrated with the hybrid system, since the ion channel coupling should *dramatically* increase the electronic signal.

3.3.1. Step 2A: nanovesicle preparation. Once the ion channel-OR hybrid membrane protein is expressed in the HEK293 cell line in step one, one can induce vesicle fusion to create small vesicles containing the membrane proteins. This can be done using cytochalasin B. The recipe was demonstrated by the Hong *et al* [22] and is spelled out here in detail:

HEK-293 cells can be incubated in DMEM containing 10 μg ml^{-1} cytochalasin B at 37 °C for 25 min in a shaking incubator. Cells can be separated by centrifugation at 500 g for 10 min. The nanovesicles can be isolated by centrifugation at 15 000 g for 30 min, then resuspended in Dulbecco's phosphate-buffered saline with 1000 ng ml^{-1} total protein density.

3.3.2. Step 2B: vesicle fusion to nanotube/nanowire devices. The vesicle fusion can be used to deposit lipid bilayers onto silicon nanowire transistors. We have successfully employed this for ion channels gramicidin (gA) and alamethicin in the past [17]. This has not been done with the ion channel OR hybrids but the principle is the same. The recipe (from our recent work) is as follows:

The suspension of nanovesicles can be filtered by a 0.2 μm nylon filter for homogeneous small unilamellar vesicles (SUVs) which helps prevent fouling effect, and improves the quality of supported lipid bilayers (SLBs). The filtered lipid suspension can be dropped into microchannels lying over nanotube/nanowire devices and incubated for 40 min at 60 °C followed by rinsing with copious 1xPBS to remove unbounded lipid bilayers.

3.3.3. Step 2C: measure electrical response to odorants (specificity). One can measure the nanowire/nanotube depletion curve, shown in figure 9. The concentration will be varied from fM to mM. The initial measurement will be a dc, time averaged measurement. We have successfully carried out this measurement for other ion channels (figure 8), and this time one can do it for the OR-K+ ion channel combination, and test for sensitivity to odorants.

3.4. Step 3: demonstrate sensitivity: single ion channel firing detection in response to a single molecule of ligand binding.

Here, the goal is to see an increase in the number of ion channels that fire and then correlate and titrate that with ligand concentration, ultimately demonstrating the electrical spike in response to binding of a single ligand molecule. One can test sensitivity to the odorants shown in figure 6 as proof of concept, as they have worked well for Hong *et al* [12] for florescence sensing of electrophysiological activity.

We divide this step into three sub-steps:

3.4.1. Step 3A: measure current spikes from opening/closing of single ion channels. One can repeat our gA experiment using K+ channels alone. This is a 'control' check to determine that K+ channel activity can be registered.

3.4.2. Step 3B: measure current spikes from opening/closing of single ion channels. We believe the ion channels will not fire unless the odorants are present so this will be a control experiment. Since in step 3A one will have demonstrated sensing of K+ channels (wild type), the anticipated null experiment will be validated (figure 10).

Note that this is somewhat analogous to a photo-multiplier tube detecting single photons: when a *single odorant molecule* binds to an OR-K+ channel complex, it

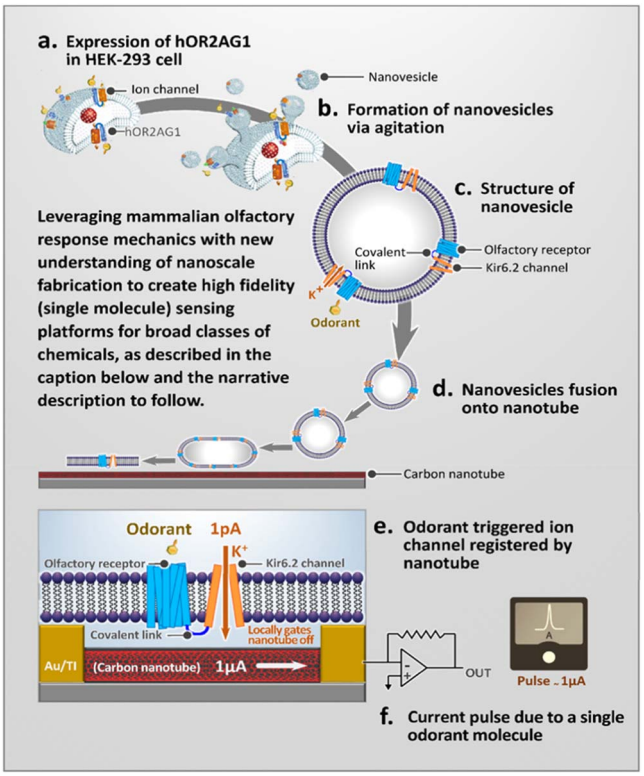

Figure 10. (a), (b): Expression of OR-Kir6.2 fused membrane proteins in HEK-293 cells, and formation of vesicles containing membrane proteins. (c) Structured nanovesicle containing olfactory receptors covalently bonded to ion channels transmit conformation changes to ion channels. (d) Vesicles fused to integrate membranes onto nanotube or nanowire devices. (e) Signal of small protein conformational changes when odorant binds to an OR, are amplified when K+ ion channel opens, locally turning off the nanotube or nanowire, and changing the nanotube or nanowire current from 1 micro A to zero. (f) Inverting amplifier reads positive pulse in response to binding a single odorant molecule.

causes the channel to open. The pA of K+ ion channel current locally turns off the nanotube or nanowire, changing the nanotube/nanowire current from 1 μA to zero. This is because the nanotube/nanowire is *extremely* sensitive to charge so even a small amount of potassium ions will gate the nanotube/nanowire completely off. Since in the on state $\sim$1 μA flows, it follows that the system displays a current gain (to sense the ion channel opening) of 1 μA/1 pA, i.e. $\sim$ one million amplicafication. After an inverting amplifier, a positive pulse is observed in response to the binding event of a single odorant molecule (see figure 1).

Because the OR is specific, it only will sense the particular odorant molecule that it has evolved to sense, and no other molecules. Hong *et al* [12] showed this already above in ensemble measurements where the net effect of many ORs acting together had a very specific response, albeit not at the single molecule level we are envisioning.

Thus, we expect *single molecule sensitivity* with specificity that can distinguish between moieties that differ by as little as one carbon atom.

3.4.3. Step 3C: determine effect of odorants on spike frequency. One can measure the spike frequency as a function of odorant concentration and composition. The main goal of this will be to show definitively that the odorants specifically cause single ion channels to fire. Once this is demonstrated, the sensitivity and specificity will be quantified.

3.5. Challenges and scientific uncertainties

We lack some real basic scientific understanding of the mechanism of interaction between the nanowire/nanotubes and the ion channel, specifically:

1) We do not have a detailed, quantitative circuit model of the effect of the ion channel current on the rest of the active device.
2) We do not control or even have any means to measure where the ion channel is either a) along the length of the nanotube/nanowire or b) on the surface of the nanotube/nanowire network or graphene device.
3) We do not know how low the open probability of the OR-K+ channel will be in the absence of analyte.
4) While we are very confident (with yield approaching 50%) of sensing ion channels by measuring what we call the 'gate current' i.e. the current from the nanotube/nanowire to the solution *through* the ion channel, our experience with measuring the nanotube/nanowire source-drain current (which is an *amplified* version of the gate current) as spikes in response to ion channels firing is very recent, so much so that we do not have any yield data for this process.
5) The sensor for an e-nose is referred to a gas phase sensor. Indeed, that is how the mammalian nose functions (sensing gas phase analytes). However, the ion mechanism in both natural nose function and this envisioned artificial fusion nano-bio nose requires fluid near the OR protein. Therefore, an important challenge for this field is to engineer a liquid layer on top of the bilayer that mimics the liquid layer in a natural nose, to enable gas phase analytes to diffuse into the liquid and be sensed by binding to the OR: 'Smell'. However, without such progress, an 'e-Tongue' is possible as well.

These scientific 'known unknowns' can and will need to be addressed to bring the electronic nose to fruition. Although one could 'swing for the fences' and attempt the single molecule, highly specific sensor based on current knowledge, it is very likely that one will need significant basic research effort to address questions #1–3 above before a significant milestone can be demonstrated, which would enable a more engineering approach towards sensing, e.g. quantify yields, rates of false positives, sensitivity, specificity, and scalability.

In addition to this cadre of scientific unknowns, there is also the challenge that each of these three steps (genetic engineering and expression of two membrane proteins fused together, nanotube/nanowire tethering of lipid bilayers able to sense single ion channel currents, and demonstration of

high selectivity as high as natural OR systems) is a major technical challenge and requires highly skilled, dedicated labor. These are not projects for beginning graduate students or short summer stints of skilled PhDs. These technical steps require significant time, effort, attention to detail, and supplies enough to anticipate multiple trials.

4. Towards massively parallel sensing

The expected outcome is the ability to sense anything the nose can, from TNT to aromatics, etc. For example, anything that can be detected by sniffing dogs could be detected if the olfactory receptors and ligands were known. This lays the scientific foundation for chemical weapons detection systems with low false positives, as well as IED detection [43, 44]. In addition, this technique lays the groundwork for developing high precision chemical detection in general which can be utilized in a variety of applications requiring high precision process control (i.e. advanced manufacturing).

Finally, since this sensor is electronic it could be integrated in the future into a massively parallel e-nose system with signal recognition based on hundreds of different OR sensors integrated onto one chip [45–47].

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

ORCID iDs

Sangjun Noh https://orcid.org/0000-0001-5948-1812
Francesco Tombola https://orcid.org/0000-0002-9315-0352
Peter Burke https://orcid.org/0000-0002-8883-1014

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