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A perspective on studying GPCR signaling with RET biosensors in living organisms

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GPCR - G-protein-coupled receptor; RET - resonance energy transfer; BRET - bioluminescence resonance energy transfer; FRET - fluorescence resonance energy transfer; CFP - cyan fluorescent protein; YFP - yellow fluorescent protein; GRK - G-protein-coupled receptor kinase; QY - quantum yield; FLIM - fluorescence lifetime imaging; MP - multiphoton, FP - fluorescent protein; PDMS - polydimethylsiloxane

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Abstract

The last frontier for a complete understanding of G-protein-coupled receptor (GPCR) biology is to be able to assess GPCR activity, interactions, and signaling *in vivo*, in real time within biologically intact systems. This includes the ability to detect GPCR activity, trafficking, dimerization, protein-protein interactions, second messenger production and downstream signaling events with high spatial resolution and fast kinetic readouts. Resonance energy transfer (RET)-based biosensors allow for all of these possibilities *in vitro* and in cell-based assays, however, moving RET into intact animals has proven difficult. Here, we provide perspectives on the optimization of biosensor design, of signal detection in living organisms as well as the multidisciplinary development of *in vitro* and cell-based assays that more appropriately reflect the physiological situation. In short, further development of RET-based probes, optical microscopy techniques and mouse genome editing hold great potential over the next decade to bring real-time *in vivo* GPCR imaging to the forefront of pharmacology.

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Introduction

The study of G-protein-coupled receptor (GPCR) signaling has a long tradition in pharmacology, and led to the development of numerous drug-classes that make up almost 30% of all marketed drugs. Initially, the signaling properties of GPCRs were studied in tissue preparations or whole organs with limited knowledge of the individual components and events that were involved in the signaling process. With the advent of molecular biology techniques, it became possible to study and manipulate individual GPCR subtypes and their signaling cascades in transfected cells. The accumulated molecular knowledge of GPCRs and their signaling cascades, together with the advances in fluorescent and luminescent proteins, allowed for the development of resonance energy transfer (RET) sensors by several groups (reviewed by Lohse et al., 2012; Marullo and Bouvier, 2007). RET, based on Förster resonance energy transfer, is the non-radiative transfer of energy from an emitting donor to an absorbing acceptor molecule. RET is perfectly suited to study intra- and inter-molecular changes in distance and/or orientation due to conformational changes and protein-protein interactions (Hamers et al., 2014). RET-based sensors permit the study of signaling events in living cells with a kinetic resolution down to the millisecond time range (Lohse et al., 2012 and references therein).

Analyses of RET-based sensors are a proven technology to study cellular processes *in vitro* or in cells. However, as discussed below, implementation for *in vivo* use has been challenging. To bridge the gap, the contrast and brightness of RET-based sensors need to be improved, advances in optical detection methods are required and realistic *in vitro* or cell-based models need to be developed that more closely resemble the physiological properties of whole organisms.

In this perspective article we briefly discuss the currently available RET-based approaches used to study GPCR signaling. More importantly, we discuss what would be desirable or required as improvements to extend the use of RET-based sensors in a more physiologically relevant *in vitro* as well as *in vivo* setting. This perspective is not meant to be exhaustive, but seeks to highlight recent breakthroughs that we judge most relevant when moving RET-biosensors into more physiologically relevant contexts.

State of the art: RET-sensors

RET-based biosensors to monitor GPCR signaling have been developed for many of the events that occur upon receptor activation, up to the level of second messenger production and downstream protein regulation (Clister et al., 2015; Lohse et al., 2012). The initial events that can be monitored are ligand binding to the receptor (Ma et al., 2014), receptor oligomerization (Angers et al., 2000), receptor activation, heterotrimeric G-protein activation, G-protein-coupled receptor kinase (GRK) and β-arrestin recruitment (Lohse et al., 2012) and intramolecular changes within β-arrestin (Charest et al.,

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2005). In general, two detection strategies are used: fluorescence- or bioluminescence-resonance-energy transfer (FRET or BRET, respectively). The major difference between these two approaches is the light source used to monitor the sensor. In case of FRET-probes, an external light source is required to excite the donor fluorophore resulting in high emission intensity allowing detection with high temporal and spatial resolution. In contrast, for BRET-probes an enzymatic substrate conversion and hence chemical light generation achieves the excitation, yielding lower levels of energy but preventing direct excitation of the acceptor. When RET takes place, the excited state energy of the donor is transferred to the acceptor, which returns to the ground state by emission of fluorescence. RET can be optically detected as a decrease in donor emission, a decrease in excited state lifetime and an increase in acceptor emission (Goedhart et al., 2014).

The most common optical read-out for RET is the ratio of acceptor emission over donor emission. Both BRET and FRET measurements have been realized for GPCRs as well as heterotrimeric G-proteins (Lohse et al., 2012). FRET and BRET probes come in different designs. Popular pairs are two genetically encoded fluorescent proteins in case of FRET (predominantly cyan and yellow variants derived from *Aequorea victoria* green fluorescent protein) or one luminescent protein (frequently luciferase from *Renilla reniformis*) and one fluorescent protein (frequently variant forms of the green fluorescent protein; GFP from *Aequorea victoria*) in case of BRET. An alternative combination has been realized by employing a small tetracysteine tag, to which a fluorescein derivative (i.e. FlAsH) can bind with high specificity as acceptor (Hoffmann et al., 2010). Other tags, like the SNAP-, CLIP- and Halo-tags, are also available (Comps-Agrar et al., 2011). These genetically encoded tags are enzymes of about 20 kDa that catalyze the covalent attachment of small (fluorescent) molecules to their protein core (Hinner and Johnsson, 2010). Finally, it is also possible to use fluorescent ligands in combination with a GPCR (tagged with either a luminescent or fluorescent protein) in BRET and FRET applications to monitor ligand binding (e.g. Albizu et al., 2010; Stoddart et al., 2015).

Both luminescent proteins and the genetic tags that can covalently bind a fluorescent probe require addition of a co-factor or ligand. This allows flexibility with respect to the choice of the co-factor or fluorescent label. However, for *in vivo* applications there is the additional challenge of ensuring that substrate molecules (e.g. luciferin for BRET or conjugated dyes for FRET) can reach their desired target (e.g. crossing the blood brain barrier). These challenges also apply for the application of fluorescent ligands that are used to probe GPCR binding. Administration needs to be optimized and the pharmacokinetic properties of the substrate or fluorescent ligand in terms of distribution and excretion need to be considered. For example, the lipophilicity of the fluorescent ligand will determine its local concentration in the immediate vicinity of the GPCR under study (Sykes et al., 2014). In addition, the ligand has to display the necessary receptor subtype selectivity to label the receptor of interest in a cell membrane that may contain other receptors for the same endogenous hormone or neurotransmitter. For example, most cells express multiple adenosine receptors, so it is important to

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develop highly selective fluorescent ligands for the specific adenosine receptor of interest (Vernall et al., 2014; Vernall et al., 2013), especially in the context of an entire organism. Pharmacokinetic considerations are also important for the use of tetracysteine tag specific FlAsH (see above) and its red-shifted variant ReAsH *in vivo*, to prevent aspecific labeling. While this technology appears to be applicable in tissue slices, it remains to be tested whether it is generally applicable in *in vivo* settings. FlAsH has been used in fly eyes (Venken et al., 2008) supporting the idea that it could become a useful tool for *in vivo* studies.

Importantly, RET-based sensors have successfully been used in cell culture experiments and have significantly extended our knowledge of agonist efficacy, GPCR function and engagement with intracellular signaling cascades (Lohse et al., 2012; Schann et al., 2013). However, to exploit their full potential and to enable *in vivo* studies on GPCR signaling, further improvements of RET-sensors are required. The biosensors developed for the measurement of second messenger production or the activation of downstream effectors such as kinases detect amplified signals compared to GPCR or G protein activation sensors and are therefore more sensitive, likely facilitating their use *in vivo*.

Optimization of RET-sensors for *in vivo* applications

Indeed, several RET-probes have already been successfully applied in living organisms. They mostly represent intramolecular FRET-reporters, such as those sensing the activation of small GTPases, the production of the second messenger cGMP and the protease activity of caspase-3 and calpain (Janssen et al., 2013; Stockholm et al., 2005; Thunemann et al., 2014; Timpson et al., 2011b). Nonetheless, an intermolecular sensor has also been described for the interaction between Cdc42 and WASp (Sharifai et al., 2014). In general, these FRET-based biosensors incorporate CFP and YFP for historical reasons, since this pair was among the first that were successfully used in a FRET-based sensor in cells. Additionally, the CFP-YFP pair provides good contrast in ratiometric FRET imaging (Goedhart et al., 2014), due to the high quantum yield (QY) of the acceptor. Finally, the weak heterodimerization of this pair strengthens the FRET efficiency in the high-FRET state.

Unfortunately, the CFP-YFP pair suffers from shortcomings, which may limit *in vivo* imaging. The extinction coefficient of CFP is relatively low and the blue excitation light (~430 nm) excites autofluorescence. The photostability of YFP is rather poor, limiting the number of photons that can be detected, and the YFP fluorescence is sensitive to pH. As excitation of the donor is not necessary, BRET does not suffer from photobleaching, phototoxicity and autofluorescence, thereby reducing background signals (Angers et al., 2000; Xu et al., 1999). Indeed, BRET sensors have been used in long-term intravital imaging of zebrafish and mice (Bakayan et al., 2014; Dragulescu-Andrasi et al., 2011). *In vivo* BRET detection was also possible using a polystyrene skin window and fiber optics to

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visualize the recruitment of β-arrestin fused to a blue-shifted GFP (GFP2) to V2-vasopressin receptor fused to *Renilla* luciferase (RLuc) in engineered cells transplanted into mouse kidney (Huang et al., 2007). β-arrestin recruitment to the β2-adrenergic receptor (β2AR) could also be detected by BRET in cells and tissue slices derived from transgenic mice expressing GFP2-βarrestin and β2AR-Rluc (Audet et al., 2010). However, due to their relatively low light output, sensitive spectrometric detection is required and high-resolution subcellular imaging studies are rendered difficult. Yet, improvements in BRET probes and microscopy equipment should soon enable BRET imaging at the subcellular level both *ex vivo* and *in vivo*. Subcellular resolution was already obtained in engineered HEK293 cells and primary cultures of hippocampal neurons (Coulon et al., 2008; Moutin et al., 2012; Takai et al., 2015). However, in living organisms, subcellular resolution has, to the best of our knowledge, not yet been reached with respect to BRET sensors and optimized BRET partners (Kamal et al., 2009) and substrates will be needed. For instance, the use of brighter versions of luminescent enzymes such as RLuc8 (Loening et al., 2006), RLuc8-S257G (Takai et al., 2015) and variants thereof like RLuc2 (Breton et al., 2010; Leduc et al., 2009) in combination with luciferase substrates such as methoxy e-Coelenterazine (Me-O-e-CTZ also known as Purple coelenterazine), which is 13 times brighter than the coelenterazine-400a (deep blue coelenterazine), will certainly contribute to shortening the image acquisition time and provide better spatial resolution.

Another avenue to increase the light output for BRET imaging would be to use the GFP from *Renilla reniformis* (RGFP) rather than from *Aequorea victoria* as the energy acceptor. Indeed, in combination with RLuc it provides a BRET pair with an optimized spatial relationship between the chromophores and a higher QY that results in much larger signals. Although the naturally occurring self-association between RLuc and RGFP may limit some of its uses, it proved not to be a problem when the 'background' affinity of the reporter system is lower than that of the 'reported' proteins under study (Molinari et al., 2008). Whether this BRET pair can be of general use and could provide higher resolution remains to be investigated.

Red and near-infrared (NIR) light shows less scattering and penetrate deeper into tissue. Therefore, many of the fluorescent ligands developed to date incorporate red fluorophores, also in order to overcome interference by auto-fluorescence. In addition, fluorophores that are environmentally sensitive (e.g. have an increased QY in a lipid environment such as BODIPY 630/650) and therefore get brighter as ligand binds to a cell surface receptor may be required *in vivo* where washout of the fluorescent ligand is not possible. Importantly, also BRET and FRET will benefit from red-shifted emission in an *in vivo* setting. Hence, RET detection would be substantially improved if bright, high QY RFPs would become available. Given recent engineering efforts that resulted in novel bright fluorescent proteins (mTurquoise2, mNeonGreen), we are confident that bright (monomeric) RFPs (QY >50%) will eventually see the light. Importantly, improving BRET sensors will require the

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development of brighter luminescence proteins in addition to those described above (e.g. Nanoluc; Hall et al., 2012; Stoddart et al., 2015), which are capable of energy transfer to red-shifted fluorophores. Recently, RFPs have been used in both BRET and FRET sensors in living organisms (Bakayan et al., 2014; Dragulescu-Andrasi et al., 2011; Keese et al., 2010), also for the study of a GPCR (Fruhwirth et al., 2011). A red-shifted unimolecular BRET sensor has also been successfully used to detect a cancer-associated antigen *in vivo* using an antibody attached to a far-red fluorescent indocyanine derivative fused to *Cypridina* luciferase via glycol chains (Wu et al., 2009), illustrating the advantages of red-shifted BRET emission for *in vivo* detection in tissue.

The above-described FRET sensors are commonly sampled based on intensity, which is complicated by the fact that e.g. the CFP signal is more scattered than the YFP signal in tissues. However, FRET-based biosensors can also be analyzed by fluorescence lifetime imaging (FLIM). A change in FRET is detected by a change in donor lifetime (Goedhart et al., 2014). This detection strategy requires a bright, photostable donor and an acceptor with good absorbance (full maturation), not necessarily high QY. However, most FRET-based biosensors show a poor dynamic range when analyzed by FLIM, making it difficult to measure subtle changes. Exceptions include protease sensors and a cAMP sensor that has been optimized for FLIM (Klarenbeek et al., 2015). A dedicated effort to engineer biosensors with high fluorescence lifetime contrast is crucial to increase our chances to successfully apply biosensors *in vivo*, as exemplified by the development of a FLIM-based sensor that enables the detection of PKA activity at the subcellular level upon endogenous GPCR activation in acute striatal slices (Chen et al., 2014). Several strategies have been reported which aim at increasing contrast (Hamers et al., 2014). While such optimization is nowadays standard for ratiometric FRET sensors, similar strategies to improve sensors for FLIM are scarce.

Since the dynamic range of RET-based sensors can be low, alternative strategies for functional imaging of GPCR signaling with high contrast are briefly highlighted, including (i) protein complementation strategies, (ii) homotypic fluorescent protein interactions and (iii) insertion of sensory modules in circularly permutated proteins. Protein complementation is a powerful strategy because of its contrast (light or no light), but the downside is that the complementation generally is irreversible, limiting its use in dynamic experiments. A recently reported reversible complementation system may represent a breakthrough in this area (Tchekanda et al., 2014). Moreover, homotypic interactions between the beta-barrels of two fluorescent proteins can lead to fluorescence changes. This was first observed between two GFP barrels and was denoted proximity imaging (PRIM). A similar system based on RFP has been reported (Alford et al., 2012). A recent extension of this strategy may be employed to qualitatively detect protein-protein interactions or conformation changes (Ding et al., 2015). Interestingly, insertion of sensory modules near the chromophore of a permutated fluorescent protein has resulted in a new generation of high contrast sensors that detect calcium *in vivo*

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(Ahrens et al., 2013). This approach can be generalized and we expect that this sensor design will be used to engineer biosensors for other processes in the future.

Certainly, also endpoint-based approaches provide valuable information and constitute a promising intermediate for looking at GPCR activation *in vivo*. For instance, S1P1 receptor activity can be detected at the cellular level in mouse tissues and embryos expressing both the S1P1 receptor fused to a transcription factor linked by a protease cleavage site and a β-arrestin/protease fusion. Upon activation of the receptor, β-arrestin is recruited, thereby leading to a release of the transcription factor that can then induce the expression of a GFP reporter gene in the nucleus (Kono et al., 2014).

Detecting RET-sensors in living organisms

In addition to the enhancement of probes and the optimization of model systems (e.g. the use of non-pigmented, hairless mice and transparent adult zebrafish), current developments in microscopy techniques and *in vivo* imaging could yield potentially exciting possibilities for efficient introduction of FRET and BRET probes in animal systems. Several of the FRET-sensors mentioned above have been imaged in living organisms using FLIM, mostly in combination with multiphoton (MP) microscopy. In MP microscopy, the simultaneous absorption of two or more low-energy infrared photons excites an electron within a fluorophore. The chance for this event to occur is low and therefore only happens in the focal point of the objective. As such, MP microscopy shows reduced phototoxicity and –bleaching, reaching an imaging depth of about 1 mm (compared to 100 μm in confocal microscopy) and inherently leads to optical sectioning. Therefore, it does not require detection pinholes and enables so-called non-descanned detection since there are no photons coming from out-of-focus areas (Timpson et al., 2011a). Importantly, this facilitates the detection of scattered photons, as the detector can be placed as close to the objective as possible. Unfortunately, the generation of infrared photons with wavelengths between 650 and 1100 nm requires an expensive pulsed Ti:sapphire laser. An important caveat with FLIM-based imaging is the loss of temporal resolution, which can be problematic for real-time measurements. Several recent advances in multiphoton microscopy provide improved acquisition speeds for FLIM-based approaches (Kirkpatrick et al., 2012; Poland et al., 2014; Rinnenthal et al., 2013). Additionally, commercially available enhancements to microscopy are now available, including gallium arsenide phosphide (GaAsP) multi-detector units, which have higher quantum efficiency and wavelength sensitivity, while still enabling low-noise high-speed imaging.

By virtue of so-called imaging windows, certain organs and tissues are surgically exposed and become accessible to RET detection (Ellenbroek and van Rheenen, 2014). Moreover, in neuroscience, cranial windows are opened and gradient-index optics (GRIN lens) are implanted into deep tissue structures,

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such that single cells can be visualized in real time, in awake behaving mice. Other reports have used fiber optic photometry approaches to detect subtle changes in calcium responses (Cui et al., 2014; Warden et al., 2014). Optimizing GRIN lens technology, coupled with advances in materials engineering have dramatically reduced the size of electronics, LEDs, and optics, while maintaining detection and resolution at cellular scales. Also, decreasing the effects of physiological motion by for instance heartbeat-triggered scanning has improved image quality. Moving deeper into a specimen brings with it more specimen-induced aberrations. Adaptive optics compensate for these aberrations by modulation of the excitation beam (Girkin et al., 2009).

Towards the imaging of GPCR signaling in organs-on-a-chip?

The above-mentioned approaches should enable the introduction of an increasing number of RET-based biosensors into living model organisms for the study of GPCR signaling. In addition to model organisms, the design of optimized *in vitro* assays has to be considered, since proper animal models are not at hand for each human physiological setting (Langenhan et al., this issue). Also, experiments in living organisms are in general not amenable to extensive manipulation, even though recent breakthroughs allow for the on-demand activation of GPCRs by light (i.e. optoXRs, Masseck et al., 2014). Moreover, in order to perform high throughput assays of e.g. drug efficiency, the development of more predictive *in vitro* assays using patient-derived cells is warranted. Besides the pharmacological benefits, these systems will also be very helpful in investigating the fundamental aspects of GPCR signaling. Especially in the field of regenerative medicine, multidisciplinary efforts have led to the development of tools (see below) that will be useful to GPCR-related research.

Classically, many signaling studies are performed using a transformed cell line cultured on two-dimensional substrates under atmospheric oxygen pressure, exogenously expressing the protein (sensor) of interest. However, the properties of a GPCR, and the signaling that results from its activation, will depend on native expression levels, cellular/membrane location (e.g. extent of cell polarity and the complexity of its membrane organization) and cell type (Corriden et al., 2013; Head et al., 2014). For this reason it is important to apply the sophisticated approaches described above to native cells. Viruses (e.g. lentivirus or adenovirus) offer approaches to deliver vectors encoding the proteins of interest to native cells. Intramolecular RET-sensors can be directed to the AAVS1 locus, a 'safe harbor' in the human genome (Smith et al., 2014). GPCR sensors are encoded by a single gene, while for heterotrimeric G-protein sensors, the α-, β-, and γ-subunit all need to be expressed at similar levels to allow optimal function. The co-expression from a single promoter ensures stoichiometric co-expression of all three subunits and results in robust detection of G-protein activation by FRET (Goedhart et al., 2011). This design simplifies the development of transgenic organisms with multimeric RET sensors.

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In order to obtain physiologically relevant expression levels, intermolecular RET-sensors can replace their endogenous counterparts by means of genome editing techniques (i.e. TALEN, ZFN or CRISPR-Cas; Gaj et al., 2013), provided that this leads to a detectable signal. Reassuringly, several knock-in mice expressing GPCRs fused to a FP under the control of an endogenous promoter have been described (for a review, Ceredig and Massotte, 2014; Scherrer et al., 2006).

Importantly, cells do not only respond to a plethora of soluble compounds, physical cues appear to be equally important in the regulation of cellular function. Both stiffness and dimensionality influence processes like cell morphology and differentiation. Focal adhesions, consisting of e.g. integrins, connect the extracellular matrix (ECM) to the actin cytoskeleton inside the cell and enable stiffness sensing. As GPCRs are regulated by and involved in the regulation of focal adhesions (Luttrell and Luttrell, 2004), it is to be expected that GPCR-mediated signaling will also be influenced by the stiffness of the substratum the cells grow on. Indeed, several GPCRs have been reported to be mechanosensors themselves (Storch et al., 2012) and for some of them the functional consequences of their mechanotransduction mechanisms are known to be relevant in pathophysiology (Cook et al., 2014). Moreover, certain adhesion GPCRs have been described to functionally interact with ECM-components like laminin (Monk et al., this issue; Petersen et al., 2015; Scholz et al., 2015). A straightforward protocol has been developed that enables high-resolution imaging of cells in three-dimensional extracellular matrices of which the stiffness can be controlled (Fischer et al., 2012). However, various other systems are available that for instance allow for cellular remodeling as the material used is sensitive to matrix metalloproteinases (Baker and Chen, 2012). The choice for a particular system clearly depends on the cell type(s) and research question to be investigated.

Recent findings have indicated that the expression levels of certain cell adhesion molecules may change depending on the oxygen level. The same holds for several proteins involved in GPCR-mediated signaling, such as GRK2 and β-arrestin-1 (Lombardi et al., 2004), RGS5 (Jin et al., 2009) and the GPCRs β_2AR (Xie et al., 2009) and CXCR4 (Romain et al., 2014). Thus, the use of physiological oxygen levels, which range between 1 and 11%, may turn out to be important in improving cell-based assays (Carreau et al., 2011). To implement this, the microscope has to be placed in a so-called glove box, thereby providing an environment in which the oxygen level can be tuned.

As one begins to investigate the use of fluorescent ligands and RET-based biosensors in native cells (Audet et al., 2010) there is also the need to consider the impact of neighboring cells on the intracellular responses being measured and whether every cell (even of the same cell phenotype) expresses the same portfolio of GPCRs. For example, in small arteries and arterioles, endothelial cells are coupled to both each other and adjacent vascular smooth muscle cells via gap junctions (Dora and Garland, 2013). In pressurized rat mesenteric arteries, calcium signaling in endothelial cells is subject

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to regulation by surrounding smooth muscle cells via myoendothelial gap junctions (Kansui et al., 2008). Three-dimensional co-culture systems have been developed in which cells cannot attach to the surface and therefore have to attach to each other. Pipetting together different cell types has been shown to result in spheroid formation consisting of mixed cell types. The recent advent of 3D printing of matrices with cells, also enabling the spatial patterning of ECM components, will lead to even more flexibility in the production of tissue- and organ-like structures (Xu et al., 2013).

Certainly, so-called organoids are in need of oxygen and nutrients. Here, microfluidic devices come into play. The flow they provide does not only mimic blood flow, thereby distributing oxygen and nutrients and removing waste products, but also simulates the shear stress experienced by many cell types, which has been shown to induce GPCR signaling (Storch et al., 2012). Moreover, microfluidics can be used to change agonist levels, create chemical gradients or apply mixtures of soluble cues to investigate their effect on GPCR-mediated signaling (Torisawa et al., 2010). Many of these devices are prepared from polydimethylsiloxane (PDMS), which is flexible and, depending on the thickness, permeable to gas. Therefore, regulation of oxygen levels becomes feasible in such devices (Thomas et al., 2011). PDMS can be coated with ECM components and is transparent, enabling microscopic analysis of FRET-probes (Yu et al., 2013). Using the above-mentioned techniques and combining them with patient-derived induced pluripotent stem cells (for a review, Bellin et al., 2012) and differentiation protocols has led to the development of ‘organs-on-a-chip’ and current progress is being made in interconnecting the available organs to obtain a model for human (patho)physiology (van de Stolpe and den Toonder, 2013).

In conclusion, the impressive speed at which novel RET-based biosensors, optical acquisition and detection methods and organs-on-a-chip techniques are being developed will enable exciting new avenues in both fundamental as well as applied research on GPCR signaling. Due to the multidisciplinary aspects, GPCR biologists will need to collaborate with researchers in the fields of optics, genetics and materials design to advance our understanding of *in vivo* GPCR biology.

Author contributions

Wrote or contributed to the writing of the manuscript: Adjobo-Hermans, M.J.W., Bouvier, M., Bruchas, M.R., Goedhart, J., Hill, S.J., Hoffmann, C., Rinken, A., Woolard, J. and van Unen, J.

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