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Structural Biology of G Protein-Coupled Receptors: new opportunities from XFELs and cryoEM

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Abstract

G protein-coupled receptors mediate cell signaling and regulate the majority of sensory and physiological processes in the human body. Recent breakthroughs in cryo-electron microscopy and X-ray free electron lasers have accelerated structural studies of difficult-to-crystallize receptors and their signaling complexes, and have opened up new opportunities in understanding conformational dynamics and visualizing the process of receptor activation with unprecedented spatial and temporal resolution. Here, we summarize major milestones and challenges associated with the application of these techniques and outline future directions in their development with a focus on membrane protein structural biology.

Introduction

G protein-coupled receptors (GPCRs) are ubiquitous cellular gatekeepers that share the characteristic architecture of a seven-transmembrane alpha-helical bundle (7TM) and are involved in the regulation of virtually every physiological process in the human body. Due to their biomedical relevance, GPCRs are targeted by a major share of therapeutic drugs and pose as attractive targets for structure-based drug design. In humans, there are over 800 receptors that belong to 5 classes: A, B, C, Frizzled, and Adhesion. Structural studies of GPCRs have been enabled about a decade ago by multiple breakthroughs in protein engineering [1-3], high-throughput nanovolume crystallization in a native-like lipidic cubic phase (LCP) matrix [4-6], and micro-crystallography [7,8]. Despite the enormous progress achieved in structural biology of GPCRs, obtaining structures of new receptors still represents a challenging task. Since GPCRs have evolved to be highly dynamic to perform

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their respective functions, their crystallization requires the stabilization in a specific conformational state. Moreover, GPCR crystals that grow in LCP are often too small for high-resolution structure determination even at modern microfocus synchrotron beamlines. Finally, our understanding of signal transduction mechanisms is incomplete without detailed knowledge about the structural dynamics of GPCRs and without structures of receptors in complex with their signaling partners, which typically are even less stable and more difficult to crystallize. These challenges of GPCR structural biology call for new tools and approaches. The recently emerged techniques of serial femtosecond crystallography (SFX) using X-ray free-electron lasers (XFELs) and high-resolution cryoelectron microscopy (cryoEM) are starting to tackle some of the most difficult problems (Figure 1).

XFELs generate extremely intense X-ray pulses of tens of femtoseconds duration with nine-to-ten orders of magnitude higher peak brilliance than third-generation synchrotrons. Such unique characteristics of XFELs prompted a new approach for crystallographic data collection called serial femtosecond crystallography (SFX) [9]. Unlike traditional crystallography, where a complete dataset is collected from a single large (or a few small) crystals, SFX data are acquired from tens to hundreds of thousands of crystals intersecting the XFEL beam in random orientations. Although each crystal is destroyed by the beam, the short pulse duration allows outrunning radiation damage and obtaining structural information from intact molecules at room temperature without the necessity of cryocooling [10]. The extremely high brightness of each XFEL pulse provides sufficient signal for the detection of high-resolution diffraction patterns from micrometer- [11] and even submicrometer-sized crystals [12]. Lastly, but arguably most importantly, the femtosecond pulse duration makes time-resolved crystallography a reality, illuminating proteins in action rather than producing static “snapshots” [13-16]. Even though time-resolved crystallography is being successfully conducted at synchrotron sources, it is mostly limited to light-induced reversible reactions at time scales longer than 100 ps, whereas femtosecond XFEL pulses provide access to irreversible transitions and fundamental chemical processes like isomerization and electron transfer [17].

In parallel to these ground-breaking XFEL developments, cryoEM of biological macromolecules has undergone a ‘resolution revolution’ [18]. The advent of direct-electron detectors with improved quantum efficiencies allowed for the correction of beam-induced motions of the specimen in vitrified ice [19]. This advancement resulted in overcoming previously perceived resolution barriers of 5-6 Å in single molecule density reconstructions. Over the last few years, near-atomic resolution maps, with which the conformations of individual amino acids could be assigned, were routinely achieved, allowing the investigation of biomolecules without the need for crystallization. In combination with the development of the Volta phase-plate, resulting in a significantly improved contrast of weak-phase objects, ever smaller molecules can be studied by cryoEM [20], immensely expanding the general scope of the technique.

In this review, we describe the progress in structural biology of GPCRs during the last 4-5 years brought about by the advancements in SFX and cryoEM (Figure 2), as well as discuss challenges associated with current applications and new opportunities related to future developments of these techniques.

Major milestones of GPCR structural studies at XFELs

Structure determination of GPCRs at XFELs has been realized by the development of special viscous media injectors [21] and new sample preparation protocols [22,23]. Such injectors allowed streaming microcrystals grown in LCP across an XFEL beam for SFX data collection. Notably, the viscous media injector greatly reduced sample consumption compared with commonly used liquid media injectors and has been shown to be suitable for the delivery of crystals of soluble proteins embedded in LCP [24] or other viscous matrices [25-27].

The LCP-SFX approach (Figure 1) was first introduced in 2013 with the high-resolution room temperature structure determination of the human serotonin 2B (5-HT_{2B}) receptor in complex with the anti-migraine medication ergotamine [11]. Compared with the structure solved by traditional cryocrystallography [28] the room temperature XFEL structure displayed a distinct distribution of thermal motions and conformations of residues that likely more accurately represent the receptor structure and dynamics in native cellular environments. LCP-SFX was subsequently applied to solve structures of the human smoothened receptor in complex with the teratogen cyclopamine [21] and of the human δ-opioid receptor bound to a bifunctional peptidic painkiller [29]. In both cases, microcrystals have shown substantially better diffraction at XFELs than their larger cryocooled counterparts at synchrotrons, enabling unambiguous placement of the corresponding ligands into the electron density.

The next important milestone was reached in 2015 (Figure 2), when the first novel GPCR structure of the human angiotensin II receptor type 1 was determined by LCP-SFX [30]. Angiotensin II is a peptide hormone that plays a major role in the renin–angiotensin-aldosterone system, and is involved in the regulation of the plasma sodium concentration and arterial blood pressure. Signaling responses to angiotensin II are mediated by type 1 and 2 angiotensin receptors (AT_1R and AT_2R). While AT_1R is primarily involved in blood pressure regulation, the function of AT_2R is much less understood, although with increasing evidence that this receptor may serve as a potential target for non-opioid treatment of neuropathic pain [31]. The 2.9 Å resolution AT_1R structure in complex with an angiotensin receptor blocker was followed by the 2.8 Å AT_2R structure bound to an AT_2R-selective ligand [32]. The structures uncovered new insights into the distinct functions of the two angiotensin receptors and provided reliable templates to facilitate structure-based drug design with improved selectivity.

One of the most dramatic examples demonstrating the advantage of XFELs was the structure determination of a major signaling complex between visual rhodopsin and arrestin (Figure 3), which, at the time, was intractable by means of traditional crystallography [33] and cryoEM [34]. Arrestin binds to activated and phosphorylated receptors, blocking G protein interaction and redirecting signaling to numerous G protein-independent pathways. It has been shown that biased ligands that direct signaling through either predominately G proteins or arrestins may have pharmacological benefits compared to balanced ligands [35]. The crystals of the rhodopsin-arrestin complex, which could not be optimized to grow beyond ~20 μm, diffracted to 7-8 Å resolution at a synchrotron, while yielding a 3.3 Å (anisotropic)

structure by LCP-SFX. The structure revealed conformational re-arrangements in arrestin and rhodopsin and the details of their interactions. Recently, re-processed data improved resolution and, in combination with extensive biochemical data, revealed combinations of phosphorylation codes for arrestin recruitment by GPCRs and possibly other proteins [36].

Another example of a macromolecular complex structure determination, which has been enabled by LCP-SFX, is the 5-HT_{2B} receptor with a selective Fab antibody fragment bound to its extracellular loops (ECLs) [37]. Monoclonal antibodies (mAbs) provide an attractive alternative to small molecules therapies [38], however, the generation of mAbs against the extracellular side of class A GPCRs is challenging due to a small area of solvent-exposed epitopes. The structure of the 5-HT_{2B}/Fab complex sheds light on the mechanisms of extracellular recognition of GPCRs by antibodies.

Finally, LCP-SFX has accelerated the structure determination of full-length receptors from non-class-A GPCRs. These receptors contain large extracellular domains (ECDs) crucial for ligand recognition and signal transduction. While initial efforts were focused on the structure determination of individual domains, the structure of the full-length receptors remained elusive due to difficulties in crystallization. Recently, extensive efforts aimed at stabilization of multidomain receptors by antibodies and designed ligands have culminated in the high-resolution structure determination of the full-length class B glucagon receptor (GCGR) and the class Frizzled smoothened receptor (Smo) at XFEL and synchrotron sources [39,40]. As in the previous examples, the room temperature XFEL structures were of higher resolution and had overall superior quality with respect to their electron density maps. While most GPCR structures were solved by molecular replacement, the recent demonstration of *de novo* phasing of a GPCR structure using the anomalous signal from sulfur atoms present in most proteins [41] opened up opportunities for structural studies of novel membrane protein families at XFELs.

First high-resolution cryoEM structures of GPCRs

The first application of direct-electron detectors combined with motion correction in cryoEM to a membrane protein was the structure elucidation of the TRPV1 channel at 3.4 Å resolution [42]. This work demonstrated that cryoEM is able to overcome difficulties traditionally associated with structural studies of membrane proteins, such as low expression yields and limited stability in detergent micelles, the reasons why many membrane proteins are often not suitable for crystallization.

Another major breakthrough in cryoEM of membrane proteins was the 3.4 Å resolution structure of γ-secretase (~170 kDa) [43], a medically important protease being the source of abnormally folded amyloid-beta fibers in Alzheimer's disease. It was the first study showing that a sub-200 kDa membrane protein with no symmetry applied in the reconstruction could be resolved to near-atomic resolution. Over the last 5 years, approximately 16% of all < 4 Å structures submitted to the Electron Microscopy Data Bank (EMDB) were derived from membrane proteins, including a variety of ion channels, transporters, enzymes, and receptors. These stats highlight a higher success rate of cryoEM for membrane proteins

compared to crystallography, in which membrane proteins contribute less than 2% of all entries.

Due to the relatively small size of GPCRs and their inherent dynamic nature, their structure determination by cryoEM has been extremely challenging. At last, in 2017, structures of two class B receptors, the calcitonin receptor (CTR) [44] and the glucagon-like peptide-1 receptor (GLP-1R) [45] in complex with their cognate G proteins have been published (Figure 3). While structures of GPCRs in complex with downstream partners are highly sought after, they are particularly difficult to solve by crystallography. In contrast, cryoEM was able to readily overcome this hurdle. Both structures were obtained in complex with their native peptide agonist, Gs protein, and a stabilizing nanobody. Using such ~150 kDa complexes did not only help with the orientation determination by increasing the molecular weight (and therefore improving the contrast), but also conformationally locked the receptor in the active state to minimize structural heterogeneity. Additionally, a Volta phase-plate was instrumental for the structure determination of the CTR/Gs complex [44].

In summary, these structures highlight the potential of cryoEM to observe relatively small isolated GPCR complexes in detergent micelles at nearly-atomic resolution. One of the most remarkable features of both studies is the use of full-length, wild-type GPCRs, which has not been possible with crystallography with an exception of the visual rhodopsin. Furthermore, both studies provided important insights into the activation mechanism of class B receptors, expanding our understanding of GPCR signaling.

Current challenges, limitations and future perspectives

As outlined above, recent breakthroughs in SFX and cryoEM have greatly advanced our structural understanding of GPCRs and other membrane proteins. While both techniques produce structural models, they have different requirements and limitations (Table 1). One of the most important advantages of cryoEM is its ability to obtain structural information from single molecules, while SFX still requires crystals, albeit much smaller than traditional crystallography. The downside of cryoEM, however, is the requirement of cryocooling the sample, which helps to reduce but does not completely overcome radiation damage. XFELs, on the other hand, can reveal structures at room temperature without detectable radiation damage effects [12]. Another important limitation for cryoEM is the minimal particle size. Although a near-atomic resolution structure of 64 kDa hemoglobin has recently been reported [20], the structure determination of < 100 kDa molecules has not yet been routinely achieved. Both cryoEM and SFX methods rely on collecting large amounts of data with typical data acquisition times of a few hours for SFX vs. a few days for cryoEM. The available SFX beamtime is, however, severely limited by the extreme cost and scarcity of large-scale XFEL facilities. In contrast, cryoEM instruments, while not inexpensive, are nonetheless affordable to major universities, research institutes, and core facilities, contributing to a much broader accessibility of this method to the structural biology community worldwide.

For structural studies, resolution is the single most important parameter that defines the amount and accuracy of information, which can be deduced for a given structure. Most

recent cryoEM structures have been determined at 3–5 Å resolution (best resolution 1.8 Å [46]), while most SFX structures are in the 1.5-3.0 Å resolution range (best resolution 1.2 Å [47]). Of the 5,908 single particle structures deposited in EMDB by March 2018, only 1% (total of 62) contain structural information beyond 3 Å. The majority (82%) of these 62 maps are virus(-like) molecules, MDa-sized complexes or other high symmetry assemblies. The remaining entries consist of 11 maps, of which 10 reconstructions applied symmetry, only 6 maps are derived from proteins smaller than 400 kDa and 3 represent structures of membrane proteins. Of the above 11, the latter 3 membrane proteins are also the only ones that have not previously been solved by X-ray crystallography. These statistics illustrate the need for further technological developments before the accuracy of cryoEM maps will become on par with crystallography and can be routinely used for applications, such as structure-based drug design.

The success of both cryoEM and SFX experiments strongly depends on sample quality, although sample requirements for these techniques are quite different. CryoEM requires single particles embedded in thin vitrified ice in random orientations and at a high concentration. The preferred orientation of molecules in ice and the degradation of delicate protein complexes during the grid preparation process pose bottlenecks in cryoEM. Typical sample preparation starts with a few microliters of purified monodisperse protein solution at 0.5-5 mg/mL concentration (Figure 1). The quality of vitrified sample depends on many factors and typically involves extensive screening of several parameters such as the grid type, buffer composition, and protein concentration. The molecule itself has to be sufficiently large and rigid to allow for orientation determination. In contrast, an ideal sample for LCP-SFX contains highly ordered micrometer-sized crystals grown in LCP at a high density. The starting point is a few dozen microliters of purified monodisperse protein solution at 10-50 mg/mL concentration. Apart from the higher protein consumption, the protein for LCP-SFX has to be stabilized in a predominately single conformational state to support crystallization, which, in the case of GPCRs, typically requires extensive protein engineering.

Finally, apart from static structures both cryoEM and SFX methods can also provide information about conformational dynamics of macromolecules. CryoEM is capable of revealing multiple equilibrium conformational states in a population of single molecules [48]. XFELs, due to their femtosecond pulse duration, allow for recording molecular movies using a pump-probe technique [13,14,49], enabling the capturing of ultrafast conformational transitions and transient states.

Conclusions

With recent breakthroughs in cryoEM and XFELs, the structural biology field is experiencing a resurgence. Most current limitations will soon be addressed if not completely resolved by the developments of instrumentation and data analysis, leading to faster data collection, higher resolution and lower molecular sizes accessible to cryoEM. At the same time, XFELs will continue tackling ever smaller crystals, which may eventually approach single molecules of a typical protein size [17].

In the case of GPCRs, we would expect an increasing number of cryoEM structures of signaling complexes with different G protein types, arrestins, kinases and other partners, as well as structures of homo- and heterodimeric receptors and receptors with large ECDs. In fact, while this review was in preparation, two additional cryoEM structures of GPCR signaling complexes, obtained with a Volta phase-plate, have been published [50,51]. We anticipate that XFELs will further contribute to the high-resolution structural coverage of the whole GPCR superfamily, help with structure-based drug design efforts, and, most importantly, should produce detailed molecular movies of signal transduction by GPCRs.

Acknowledgments

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Highlights

- Recent advancements in cryoEM and XFELs accelerated structural studies of GPCRs
- XFELs enabled room-temperature damage-free structures from micrometer-sized crystals
- CryoEM demonstrated its potential for elucidating GPCR signaling complexes
- Both cryoEM and XFELs promise to shed light on structural dynamics of GPCRs

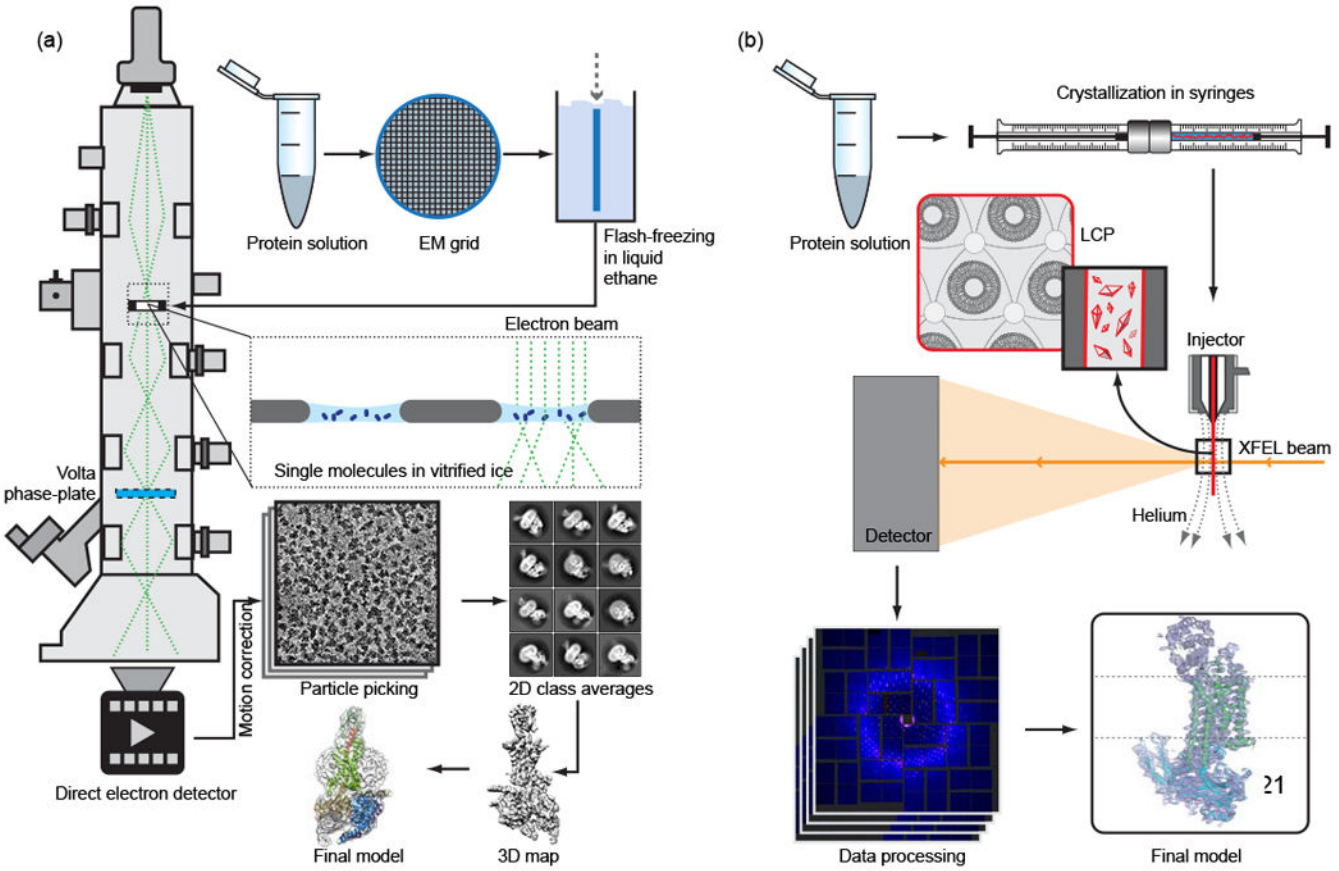

Figure 1. Schematic diagrams of cryoEM and LCP-SFX experiments

(a) For cryoEM, the purified monodisperse protein solution is deposited on EM grids, blotted and flash-frozen in liquid ethane. The grids are then cryo-transferred into the electron microscope and thousands of images are collected by a direct-electron detector. After performing motion correction, individual particles are picked and 2D classification and 3D classification is applied. Finally, a 3D map is reconstructed, which is used to fit and refine a structure model. Images from Ref. 44 have been re-used in this illustration with permission from Macmillan Publishers Ltd. (b) For LCP-SFX, purified protein is reconstituted in LCP, and crystallization is set up in syringes. After microcrystals have grown, samples from several syringes are consolidated and transferred into a viscous media injector. Tens to hundreds of thousands of diffraction images are collected from microcrystals intersecting the XFEL beam in random orientations. After data processing with specialized software, the structure is solved and refined by standard crystallographic approaches.

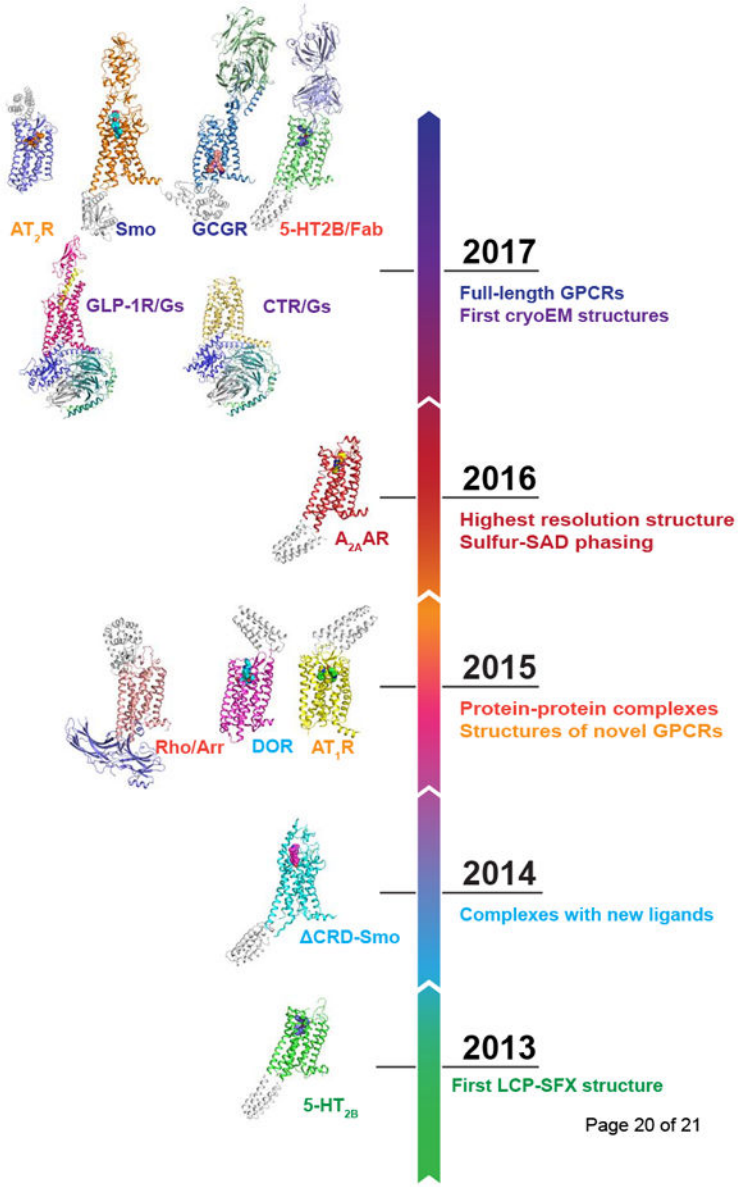

Figure 2. Timeline of major milestones (right) and published GPCR structures (left) achieved with XFELs and cryoEM

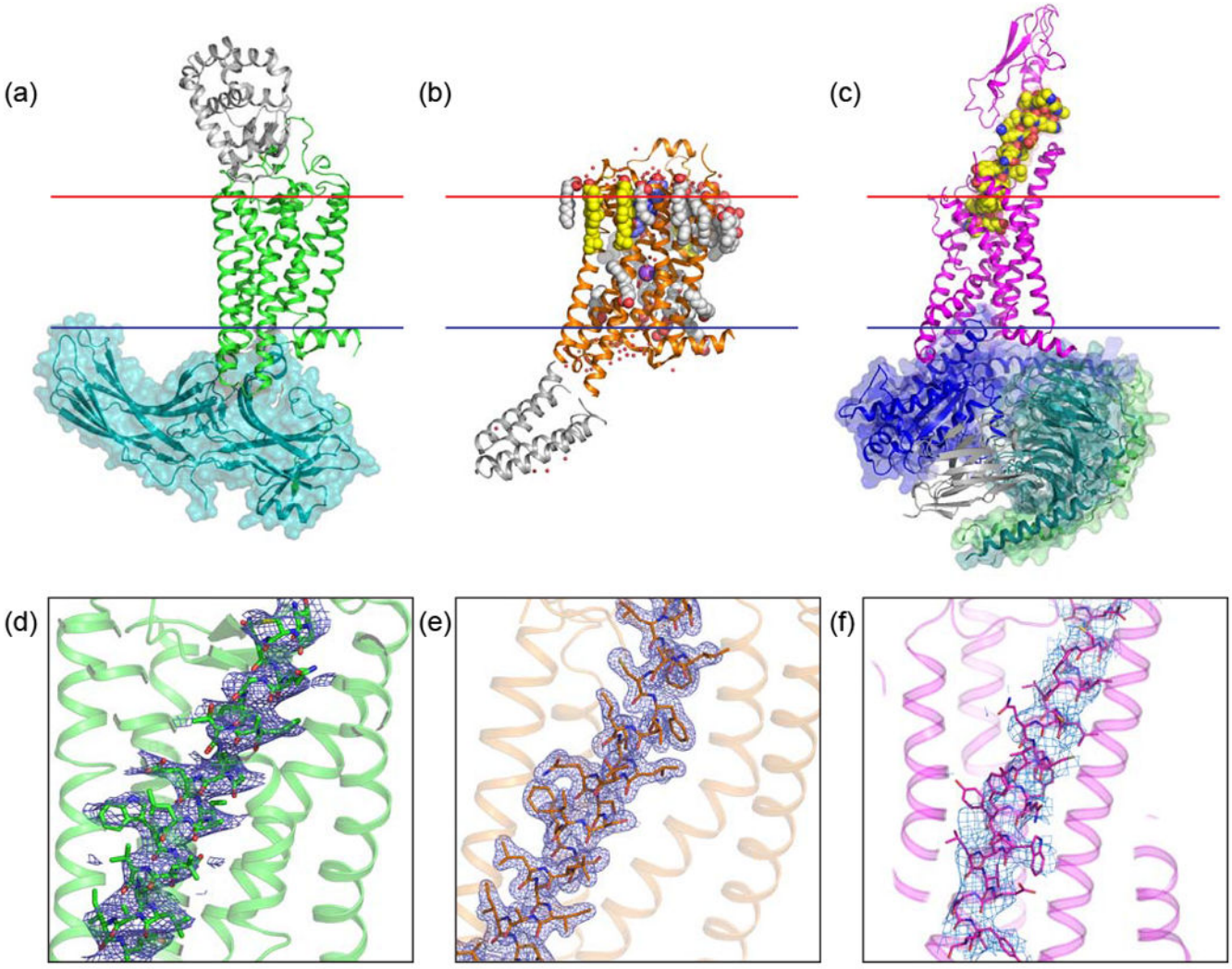

Figure 3. Examples of GPCR structures determined by LCP-SFX and cryoEM

(a) Structure of the rhodopsin-arrestin complex (PDB ID 5W0P) solved using LCP-SFX at 3.0 Å (anisotropic) resolution. (b) The 1.9 Å resolution structure of the adenosine A_{2A} receptor (PDB ID 5K2C) solved by the sulfur single anomalous dispersion (S-SAD) method using data collected by LCP-SFX. (c) The 4.1 Å resolution structure of the GLP-1 receptor in complex with its native agonist peptide, Gs protein and a stabilizing nanobody (PDB ID 5VAI) obtained by single-molecule cryoEM. All structures are shown in cartoon representation with fusion partners and the nanobody colored in gray. Transparent surface is shown for signaling partners, arrestin, and Gs protein. Ligands, lipids and ions are shown as van der Waals spheres, water molecules as small red spheres. The membrane boundaries are shown as red (extracellular) and blue (intracellular) lines. (d) – (f) Electron density of transmembrane helix III is shown as a blue mesh for corresponding structures in (a) – (c). In (d) and (e) 2Fo-Fc density is contoured at 1s level, in (f) the density is contoured at the authors' recommended level of 0.055 [44].

Table 1

Typical experimental conditions, challenges, and opportunities for cryoEM and SFX

	cryoEM	**SFX**
Temperature	Cryogenic	Room
Sample state	Isolated molecules	Crystals
Protein size, kDa	> 100 (smallest 64)	No limit
Radiation damage	Yes	No
Resolution, Å	3 – 5 (best 1.8)	1.5 – 3 (best 1.2)
Protein consumption, μg	1 – 10	100 – 500
Final protein concentration, mg/mL	0.5 – 5	10 – 50
Data collection time, hrs	24 – 96	2 – 6
Accessibility	Core facilities, in-house instruments	Large facilities: LCLS, SACLA, EuXFEL, PAL, SwissFEL
Protein dynamics	Equilibrium states in a population	Room temperature fluctuations, molecular movies, ultrafast dynamics, non-equilibrium states
Opportunities for GPCRs	Signaling complexes, homo- and heterodimers, receptors with large ECDs	Small crystals, room temperature structures, SBDD, receptor activation movies