Microscopy and spectroscopy approaches to study GPCR structure and function

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Abstract

The GPCR signalling cascade is a key pathway responsible for the signal transduction of a multitude of physical and chemical stimuli, including light, odorants, neurotransmitters, and hormones. Understanding the structural and functional properties of the GPCR cascade requires direct observation of signalling processes in high spatial and temporal resolution with minimal perturbation to endogenous systems. Optical microscopy and spectroscopy techniques are uniquely suited to this purpose because they excel at multiple spatial and temporal scales and can be used in living objects. Here, we review recent developments in microscopy and spectroscopy technologies which enable new insights into GPCR signalling. We focus on advanced techniques with high spatial and temporal resolution, single-molecule methods, labelling strategies, and approaches suitable for endogenous systems and large living objects. This review aims to assist researchers in choosing appropriate microscopy and spectroscopy approaches for a variety of applications in the study of cellular signalling.

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Abstract

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Abbreviations

2P – two-photon

AFM – Atomic force microscopy

β2AR - β_2 adrenergic receptor

cpFP – Circularly permuted fluorescent protein

DMR – Dynamic mass redistribution

DNA-PAINT – DNA point accumulation in nanoscale topography

FCCS – Fluorescence cross-correlation spectroscopy

FCS – Fluorescence correlation spectroscopy

FP – Fluorescent protein

FRET-FLIM – FRET with fluorescence lifetime imaging microscopy

LSFM (SPIM) – Light sheet fluorescence microscopy (Selective plane illumination microscopy)

mGluR – Metabotropic glutamate receptor

MINFLUX – Minimising fluorescence fluxes nanoscopy

PALM – Photoactivated localisation microscopy

PCH-FIDA – Photon counting histogram – fluorescence intensity distribution analysis

RASTMIN – Single-molecule localisation by raster scanning a minimum of light

RESI – Resolution enhancement by sequential imaging

RET – Resonance energy transfer

SIM – Structured illumination microscopy

SMLM – Single-molecule localisation microscopy

SPT – Single particle tracking

SRN – Super-resolution nanoscopy

SRRF – Super-resolution radial fluctuations

STED – Stimulated emission depletion microscopy

STORM - Stochastic optical reconstruction microscopy

TIRF – Total internal reflection fluorescence microscopy

TR-FRET – time-resolved FRET

Introduction

The GPCR signalling cascade is a major pathway of cellular signal transduction and receptors play a key role in it. GPCR signalling is responsible for a multitude of physiological processes including eyesight, heartbeat,

kidney excretion, the action of synapses, and many others. The malfunctioning of GPCR signalling is a cause of multiple diseases, including schizophrenia, heart failure, and multiple forms of cancer. Therefore, the GPCR pathway is a major drug target. Specifically, GPCRs that perceive extracellular stimuli are the primary targets of pharmacological compounds. Accurate and precise understanding of the spatiotemporal signalling properties of the GPCR cascade is of utmost importance for the development of better treatments for GPCR-related diseases.

Microscopy and spectroscopy techniques provide insights into multiple aspects of cellular signalling at a wide range of spatial and temporal scales. Individual stages of the GPCR signalling cascade vary greatly depending on the nature of the ligand, GPCR itself, expression levels, the cellular context, presence, and abundance of regulatory proteins, G proteins, and effector types (Figure 1). Signalling events span timescales from femtoseconds (Schoenlein et al., 1991) for the initial steps in rhodopsin activation to tens of minutes for gene expression regulation and the endosomal "second wave" of signalling (Tsvetanova et al., 2015). The spatial scale of signalling events spans the range from ångstroms for molecular conformational changes through micrometres for redistribution of signalling molecules in the cell to millimetres and more for the transport of hormones and neurotransmitters within the body. Therefore, techniques for the study of GPCR signalling span the entire range from femtosecond spectroscopy and the tracking of individual molecules to long-term light sheet imaging of whole living objects. For the sake of clarity, we will focus on optical microscopy and spectroscopy techniques with high spatial and temporal resolution, primarily spanning the spatial range from nanometres to micrometres and the temporal range from microseconds to seconds.

Imaging GPCR signalling in high resolution

When considering a high resolution, we need to distinguish between high spatial and high temporal resolution and techniques which excel in one or the other of these domains or provide a good combination of both (Figures 2 and 3, Supporting Table 1). The high spatial resolution required for GPCR signalling ranges from ångstroms to a few tens of nanometres (Figure 1b) while high temporal resolution ranges from microseconds to a few hundred milliseconds (Figure 1a) if we omit the initial photophysical events in rhodopsin activation. The group of techniques that primarily excel in spatial resolution can be broadly referred to as super-resolution nanoscopy. Imaging techniques which provide the best temporal resolution are mostly based on resonance energy transfer (RET), single-particle tracking (SPT), or fluorescence fluctuations. Recently, several highly promising techniques enabling a combination of high spatial and temporal resolution have emerged.

Super-resolution nanoscopy (SRN)

The spatial resolution of a conventional fluorescence microscope is restricted by the diffraction limit, which is usually 200-300 nm in the lateral direction and 500 nm in the axial direction. However, several techniques use different optical phenomena to break the diffraction limit (Figures 2 and 3). At present, the most often used are stimulated emission depletion microscopy (STED), photoactivated localisation microscopy (PALM), stochastic optical reconstruction microscopy (STORM), structured illumination microscopy (SIM), DNA point accumulation in nanoscale topography (DNA-PAINT), and super-resolution radial fluctuations (SRRF). However, several highly promising techniques enabling the achievement of subnanometre resolution have recently emerged, including minimising fluorescence fluxes nanoscopy (MINFLUX), minimal STED (MINSTED), single-molecule localization by raster scanning a minimum of light (RASTMIN), resolution enhancement by sequential imaging (RESI), and expansion microscopy.

3.1 STED

The STED technique, originally developed in the laboratory of Stefan Hell (Hell & Wichmann, 1994), achieves improved lateral and axial resolution by depleting the fluorescence emission from all molecules in a defined area except for a tiny spot less than 50 nm in the lateral direction and less than 100 nm in the axial direction (Figure 2a). In GPCR signalling research, STED has been used to determine precise localisation and evaluate clustering of cannabinoid receptor type 1 in neurons (Li et al., 2020) and uncover the distribution of endogenous glucagon-like peptide-1 receptor molecules (Ast et al., 2020). A detailed protocol for the use of ground state depletion microscopy (GSDM), a variant of STED for GPCR studies, is described in (Caetano

Crowley et al., 2019). Limitations of STED include the high laser powers required for the efficient fluorescence depletion of molecules and relatively slow image acquisition. New approaches, including MINFLUX and MINSTED, described below, allow for overcoming these limitations.

3.2 PALM and STORM

PALM and STORM techniques published just one day apart (Betzig et al., 2006; Rust et al., 2006), often referred to as single-molecule localization microscopy (SMLM) or "blinking techniques", share the same principle of selectively exciting a small number of fluorescent molecules in the sample (Figure 2b). PALM relies on the photoswitching of fluorescent proteins (FPs), while STORM relies on the blinking of organic dyes. The precise localisation of spatially separated molecules by these techniques is determined by the deconvolution of a point spread function (PSF) from the raw imaging data. This allows the achievement of a resolution of a few tens of nanometres. PALM and STORM have been used to study the nanoscale organisation of GPCR signalling components in cellular compartments and GPCR oligomerisation (Dudok et al., 2015; Jonas et al., 2015; Moller et al., 2020; Scarselli et al., 2012; Siddig et al., 2020). SMLM techniques allowed the detection of cytoskeleton-dependent β_2-adrenoceptor (β2AR) clusters (Scarselli et al., 2012), and the accurate localisation of metabotropic glutamate receptors (mGluRs) (Siddig et al., 2020) and cannabinoid receptor type 1 (Dudok et al., 2015) in synaptic zones. SMLM has also been used to detect and quantify GPCR oligomers with PALM (Jonas et al., 2015) and direct STORM allowing the determination of the exact oligomer composition (Moller et al., 2020). An interesting application of PALM combined with SPT allowed imaging of GPCR mobility with single-molecule precision in a dense environment typically inaccessible for SPT measurements (Eichel et al., 2018). Conveniently, SMLM probes can be attached not only to proteins of interest but also to small molecules, such as GPCR ligands, as has been shown for the corticotropin-releasing hormone type 1 receptor (Szalai et al., 2018), which makes these techniques very versatile and suitable for the study of endogenous systems. The main limitations of SMLM include slow data acquisition rates, localisation artefacts, large amounts of required data for processing, and time-consuming analysis. However, SMLM can be combined with photoswitching fingerprint analysis or polarisation microscopy to bypass some of these limitations.

3.3 DNA-PAINT

The DNA-PAINT technique utilises the transient binding of fluorescently labelled DNA oligonucleotides ("imager") to their complementary target strands ("docking") to achieve the blinking effect of SMLM techniques (Schnitzbauer et al., 2017) and obtain a resolution of 10 nm (Figure 2c). DNA-PAINT has been used to determine the amount and distribution of αV integrin accumulation regulated by the purinergic $P2Y_2$ receptor in pancreatic ductal adenocarcinoma (Tomas Bort et al., 2023). A modification of DNA-PAINT, quantitative PAINT (qPAINT), allows accurate molecular counting and was used for the quantitation of the $P2Y_2$ oligomer composition (Joseph et al., 2021). DNA-PAINT limitations include complex probe design, efficient delivery of the imager DNA nucleotides to the docking target, and slow data acquisition rates. However, DNA-PAINT is compatible with other SRN techniques, such as RESI, allowing the achievement of even higher resolution, making it a technique of choice for many applications.

3.4 SIM

SIM is an SRN modality that achieves spatial resolution enhancement by projecting light patterns over a fluorescent sample (Figure 2e) (Gustafsson, 2000). The known structure of such patterns allows for efficient deconvolution of the PSF and improved lateral resolution to 120 nm. Although the resolution improvement achieved with SIM is not as high as that achieved with STED and SMLM techniques, its main advantage is the ability to obtain fast imaging of dynamic processes and high compatibility with the imaging of live samples. In GPCR research, SIM has been used to localise βArrestin-2 in the membrane inside and outside of clathrin-coated pits (Eichel et al., 2016) and detect the differential segregation of activated β2AR molecules phosphorylated by GPCR kinases (GRKs) and protein kinase A (Shen et al., 2018). SIM can be used with a variety of fluorescent sensors, which increases the flexibility of the experimental system design (Wang et al., 2019).

3.5 SRRF

In contrast to other SRN techniques, SRRF requires no specialised hardware or biological probes and achieves resolution enhancement to the level below 100 nm by analysing radial fluctuations of multiple (hundreds) images with a low signal-to-noise ratio (Gustafsson et al., 2016). SRRF can be implemented using regular FPs and fluorescent dyes on standard confocal, total internal reflection fluorescence (TIRF), and even widefield microscopes. Enhanced SRRF (eSRRF), a recent technique update, enables the achievement of improved resolution in 3D (Laine et al., 2022). So far, in studies of GPCR signalling, SRRF has been used primarily as a supporting technique to visualise actin filaments alongside SPT of β2AR-βArrestin-2 complexes (Grimes et al., 2023) or determine the cellular localisation of endogenous glucagon-like peptide-1 receptor using fluorescent peptide antagonists (Ast et al., 2020). Importantly, SRRF and eSRRF are available as free ImageJ plugins. These image processing tools show great potential to democratise the implementation of SRN in a variety of applications without the need for costly equipment and specialised experimental design.

3.6 Expansion microscopy

Expansion microscopy is an unusual SRN approach that uses the physical expansion of a sample by synthesising a swellable polymer network inside it (Figure 2f) (Chen et al., 2015). Imaging of such expanded samples alleviates some of the limitations of conventional microscopes and leads to lateral and axial resolution improvement to several tens of nanometres. Expansion microscopy has been used to map the molecular organisation of the lateral hypothalamus in mice (Wang et al., 2021) and accurately detect melatonin receptor 1 localisation in the outer membrane of the brain mitochondria (Suofu et al., 2017). An intriguing recent study combined expansion microscopy with SRRF to achieve a dramatic increase in lateral resolution below 10 nm (Shaib et al., 2023). Although expansion microscopy is in principle incompatible with living samples, its reliance on non-optical methodology for the achievement of resolution enhancement may be beneficial for the study of dense samples with high expression of the labelled molecules or potentially even as a structural biology technique.

3.7 Emerging SRN techniques (MINFLUX, MINSTED, RASTMIN, RESI)

Several SRN techniques have emerged recently that can achieve resolution on the scale of the size of fluorescence emitters (Figures 2 and 3, Supporting Table 1). These techniques bear the potential to yield the resolution of a few nanometres or even several Angstroms in 3D and bridge the gap between optical microscopy, electron microscopy, and structural biology techniques. Emerging SRN techniques are challenging to implement and require subnanometer probes and subnanometer sample position stabilisation. However, they can yield combined structural and dynamic characterisation of interactions between signalling proteins and can even accurately identify conformational changes within individual proteins with nanometre resolution in living cells. The most promising techniques include MINFLUX, MINSTED, RASTMIN, RESI, and photoswitching fingerprint analysis.

3.7.1 MINFLUX, RASTMIN, and MINSTED

Minimising fluorescence fluxes nanoscopy (MINFLUX) utilises a principle somewhat similar to that of STED. However, instead of quenching fluorophores outside the central excitation point, MINFLUX uses an excitation PSF with zero intensity in the centre that makes subnanometer searching steps around the emitting molecule to minimise its fluorescence (Figure 2d) (Gwosch et al., 2020). This allows the use of much lower laser intensities compared to STED and achievement of a spatial resolution of 1-3 nm in 3D and a temporal resolution of 100 μs compatible with very accurate SPT. MINFLUX possesses enormous potential and currently its implementation is primarily limited by the high cost of the required equipment. MINFLUX can be combined with other SRN techniques including DNA-PAINT (Ostersehlt et al., 2022) or single-molecule resonance energy transfer (Cole et al., 2023) further expanding its capabilities. RASTMIN aims to alleviate the equipment cost issue of MINFLUX by using the raster scanning of a sample with spatially modulated light comprising a local minimum of intensity (Masullo et al., 2022). RASTMIN can be implemented on regular scanning microscopes (confocal or two-photon (2P)) and provides a 3D spatial resolution comparable to MINFLUX. In MINSTED, another MINFLUX competitor, the STED beam is used not to separate fluo-

rophores, which are photoswitched independently, but to find the fluorophore position (Weber et al., 2021). MINSTED provides a similar spatial resolution to MINFLUX, but its advantages include lower background noise and the ability to attenuate the desired resolution of the system. The true potential of MINFLUX, RASTMIN, and MINSTED is yet to be fully revealed.

3.7.2 Photoswitching fingerprint analysis

Time-resolved detection with photoswitching fingerprint analysis takes advantage of the finding that the photoswitching kinetics of fluorophores depend on the distance between them (Helmerich et al., 2022). Therefore, the time-resolved detection of photoswitching can increase the achievable resolution of techniques such as dSTORM to a level below 10 nm.

3.7.3 RESI

RESI is the first SRN technique to boast a subnanometer resolution (Reinhardt et al., 2023). The principle of RESI is based on sequential DNA-PAINT imaging with fluorophores of different colours and re-application of the concept of localization to super-resolution data. RESI gets close to the resolution of cryo-electron microscopy, but the true capabilities of this technique have yet to be thoroughly tested.

An important aspect of recent developments in SRN is that the size of the fluorescent marker and its localisation have become highly critical to obtaining accurate results. FPs and self-labelling systems, such as SNAP-tag or HaloTag, have become unsatisfactorily large for the desired spatial resolution. Distantly located fluorescent labels do not accurately report the localisation of the protein of interest. Therefore, genetic code expansion with unnatural amino acids, ligand-directed labelling, and the utilisation of subnanometer tags (Arsic et al., 2022; Mihaila et al., 2022) will likely become the mainstream for the future development and application of SRN techniques.

4. Resonance energy transfer (RET) techniques

FRET and BRET have been used for studies of GPCR signalling for more than two decades. RET techniques are based on the non-radiative transfer of energy from an excited fluorophore (donor) to another, closely located (2-10 nm), fluorophore with lower excitation energy (acceptor) which emits fluorescence (Förster, 1948). RET can be used for the detection of molecular proximity and conformational changes within a molecule at high temporal resolution. FRET and BRET have been reviewed multiple times elsewhere (Kauk & Hoffmann, 2018; Kroning & Wang, 2022; Olsen & English, 2023). Here we describe advanced RET applications, single-molecule, time-resolved, and fluorescence lifetime imaging FRET, which allow the observation of protein conformational changes and signalling dynamics in living samples with molecular resolution and structural insights (Figure 2 and 3, Supporting Table 1).

4.1 Single-molecule FRET (smFRET)

SmFRET detects energy transfer between individual molecules of donor and acceptor fluorophores and provides both structural and dynamic insights into intramolecular conformational changes and intermolecular interactions (Figure 2g). In the last decade, smFRET has been extensively used for studies of GPCR signalling (Quast & Margeat, 2019). SmFRET was used in multiple studies to determine the dynamics of interactions of GPCRs, particularly β2AR and A2A adenosine receptors, with different ligands and subsequent activation of G proteins (Fernandes et al., 2021; Gregorio et al., 2017; Lamichhane et al., 2015). Furthermore, smFRET allowed the detection of submillisecond conformational dynamics of the A2A adenosine receptor (Maslov et al., 2023). In live cells, smFRET was used to determine the abundance of dimers and their spatiotemporal dynamics for the μ-opioid receptor, secretin receptor, and mGluR2 (Asher et al., 2021). Furthermore, smFRET uncovered the mechanisms of β-arrestin1 autoinhibition, binding to GPCRs, and activation (Asher et al., 2022). Recent technical advances have enabled the use of genetic code expansion and click chemistry for the introduction of labels for smFRET (Han et al., 2021) as well as four-colour smFRET measurements in live cells (Sotolongo Bellon et al., 2022). The limitations of smFRET include the requirement for two labels, probe photobleaching, sophisticated labelling procedures, a limited range of usable protein concentrations, and complex data analysis. Future developments of smFRET, particularly in combination with SPT and

SRN techniques, such as MINFLUX (Cole et al., 2023), live-cell imaging, and specific direct labelling with small fluorophores (Banerjee et al., 2022), will allow the study of GPCR signalling dynamics in systems closer to natural with even higher temporal resolution.

4.2 Time-resolved FRET (TR-FRET)

TR-FRET is a spectroscopy technique that combines the detection of energy transfer with the measurement of fluorescence emission lifetimes (Ergin et al., 2016). It removes background noise from other components of the system and enables performing ratiometric measurements. TR-FRET is often used with lanthanides that exhibit large Stokes shifts, long fluorescence lifetimes, and allow for efficient multiplexing. Recently developed lanthanide complexes (CoraFluors) have high synthetic accessibility and compatibility with most existing tracers (Payne et al., 2021). TR-FRET is most often used to characterise binding events and determine binding affinities (Raich et al., 2021). In studies of GPCR signalling, TR-FRET has been applied to detect vasopressin, oxytocin, and dopamine receptor oligomers in native tissues (Albizu et al., 2010) and characterise GPCR hetero-oligomers using ligands specifically binding to such complexes (Heuninck et al., 2019). In combination with smFRET and lanthanide RET, TR-FRET can be applied to detect conformational changes in GPCRs, particularly class C, such as mGluRs (Lecat-Guillet et al., 2023).

4.3 FRET with fluorescence lifetime imaging microscopy (FRET-FLIM)

FRET-FLIM is an imaging technique, similar in principle to TR-FRET, that provides spatially resolved data (Figure 2h). The lifetime of emitted fluorescence provides a well-defined characteristic feature that can be used to identify the source of detected photons and the environment in the sample (Liput et al., 2020). FRET-FLIM allows accurate quantification of FRET measurements, eliminating reliance solely on widely varying fluorescence intensities. FRET-FLIM has been applied to detect the distribution of the dopamine receptor type 2 and the Gi proteins in membrane nanodomains (Polit et al., 2020) and uncover the plasma membrane-localised homodimers of the GPR17 receptor that controls the central nervous system myelination (Yang et al., 2020). FRET-FLIM has also been used for the detection of transient signalling processes such as cAMP degradation by phosphodiesterases (Harkes et al., 2021). Importantly, FRET-FLIM is compatible with deep tissue imaging with 2P microscopy and can be used for the quantitative analysis of endogenous GPCR signalling in brain slices (Chen et al., 2014).

5. Single-particle tracking

Ensemble imaging techniques often require overexpression of the fluorescently labelled protein of interest and cannot provide insights into the behaviour of individual molecules and the degree of variability between them. In contrast, SPT acquires information at the resolution of individual molecules (Figure 2i). SPT uses diffraction of the emitted fluorescence from a fluorescent molecule to accurately determine its subpixel localization in the image and advanced image processing to track its movement with a millisecond temporal resolution. SPT can work at low expression levels that often occur in endogenous systems and achieves the multiplexing of signals through simultaneous tracking of different fluorophores (Sotolongo Bellon et al., 2022). The limitations of SPT include its limited usability for molecules localised outside of the plasma membrane, demanding sample preparation, complex data analysis, and potential underexpression of the target protein below the expected endogenous level. SPT is commonly performed using Total internal reflection fluorescence (TIRF) microscopy, which achieves high contrast by selectively exciting only fluorophores localised in the close vicinity (typically 80-150 nm) to the coverslip surface (Axelrod, 1981). TIRF is prominently suitable for imaging molecules localised in the plasma membrane, and therefore TIRF and SPT are widely used for the study of GPCR signalling.

In the research of GPCR signalling components, SPT has been used to detect their molecular mobility (Calebiro et al., 2013; Petelák et al., 2023; Rosier et al., 2021; Sungkaworn et al., 2017), localisation (Bondar et al., 2020; Eichel et al., 2018; Gormal et al., 2020; Grimes et al., 2023; Sungkaworn et al., 2017), the dynamics of intermolecular interactions (Grimes et al., 2023; Sungkaworn et al., 2017), and di-oligomerisation (Calebiro et al., 2013; Kasai et al., 2018; Latty et al., 2015; Moller et al., 2020). SPT has been used to uncover the modes of interaction between GPCRs and β-arrestins and the dynamics of accumulation of these signalling molecules

in membrane domains, particularly in clathrin-coated pits (Bondar et al., 2020; Eichel et al., 2018; Grimes et al., 2023). SPT results have led to the concept of "hot spots" postulating the compartmentalisation of GPCR signalling into confined areas defined by the cytoskeleton in the plasma membrane (Sungkaworn et al., 2017). SPT can be combined with other techniques such as smFRET (Asher et al., 2021) and FLIM-FRET (Graham et al., 2022) in living cells to verify detected protein-protein interactions. The further automation of data acquisition and analysis for SPT (Yasui et al., 2018) will improve its experimental throughput rates. Recently developed fluorescent GPCR ligands with subnanomolar affinity compatible with SPT (Gentzsch et al., 2020; Isbilir et al., 2020; Rosier et al., 2021) possess a high potential for imaging endogenous GPCRs in living cells. Overall, SPT will likely find more applications in the imaging of conformational dynamics, component localisation, and protein-protein interactions in endogenous signalling systems.

6. Fluorescence correlation spectroscopy (FCS)

FCS is a group of techniques that analyse the fluctuations in fluorescence intensity of labelled molecules within a small observation volume (Figure 2j). FCS techniques provide information about diffusion times, molecular interactions, and concentration within the observed sample with high sensitivity and temporal resolution. FCS drawbacks include limited spatial resolution since it relies on the minute observation volume, susceptibility to photobleaching and phototoxicity effects, and the requirement for low concentrations of fluorescently labelled molecules, which present a challenge for certain proteins.

FCS approaches can be broadly divided into single-point and imaging (Figures 2 and 3, Supporting Table 1). Single-point FCS uses a fixed detection volume and does not provide spatial resolution beyond that. Single-point FCS can be multiplexed by simultaneously tracing multiple molecular species (fluorescence cross-correlation spectroscopy (FCCS)) and expanded with photon counting histogram – fluorescence intensity distribution analysis (PCH-FIDA). FCS and FCCS have been used to characterise the ligand-binding properties of multiple GPCRs (Antoine et al., 2016; Grime et al., 2020; Rico et al., 2019; Rose et al., 2012). PCH-FIDA has been used in the study of mobility and oligomerisation of several GPCRs, including serotonin, adrenergic, muscarinic, and dopamine receptors (Herrick-Davis et al., 2013). In contrast to single-point FCS, imaging FCS scans the sample and achieves the spatial resolution relevant for live-cell imaging. Imaging FCS and Raster scanning correlation spectroscopy (RICS) (Digman & Gratton, 2009) have been used to determine the diffusion times of GPCRs and G proteins (Foust & Piston, 2021) and the dynamics of bradykinin receptor signalling complexes (Philip et al., 2007) in live cells. Further, comprehensive information on single-point and imaging FCS approaches and applications in GPCR studies can be found in (Briddon et al., 2018; Kilpatrick & Hill, 2021).

FCS can be combined with other imaging modalities including TIRF, STED, and light sheet fluorescence microscopy (LSFM). Total internal reflection FCS (TIR-FCS) combines evanescent wave excitation with fluorescence correlation spectroscopy, allowing the study of molecular diffusion and interactions near the plasma membrane with high spatial and temporal resolution (Lieto et al., 2003). STED-FCS combines STED microscopy with FCS to achieve super-resolution imaging and measurement of molecular diffusion and interactions with the nanometre spatial resolution (Sezgin et al., 2019). It utilises a STED laser to narrow the effective PSF, allowing for highly localised fluorescence excitation and precise correlation analysis. Selective Plane Illumination Microscopy FCS (SPIM-FCS) combines the advantages of LSFM and FCS techniques, allowing for non-invasive three-dimensional measurements of molecular diffusion and interactions in live cells or tissues with high spatiotemporal resolution (Wohland et al., 2010). Further FCS development, particularly in combination with SRN techniques, will provide dynamic insights into signalling processes at very high temporal and spatial resolution.

7. Polarisation microscopy

The excitation probability of most existing fluorophores is sensitive to the excitation light polarisation. Therefore, fluorophore excitation and fluorescence emission when using linearly or circularly polarised light depend on molecular orientation and yield information on protein conformation and protein-protein interactions, particularly in membrane-localised systems (Figure 2k) (Lazar et al., 2011). This phenomenon has

been used to determine the extent of dissociation of heterotrimeric G proteins upon activation (Bondar & Lazar, 2014) and the detection of pre-existing complexes between GPCRs and G proteins (Bondar & Lazar, 2017). Recent advances in the characterization of fluorescent proteins (Myskova et al., 2020) enabled quantitative structural insights into membrane protein conformations using modulation of the excitation light polarisation (Bondar et al., 2021; Rimoli et al., 2022). Moreover, polarisation microscopy has been combined with STORM for the superresolution imaging of dense samples (Rimoli et al., 2022). Polarisation microscopy remains an underutilised tool, likely due to the relatively complex implementation and data analysis, but it holds great promise in expanding the capabilities of other available imaging techniques. Importantly, fluorophore sensitivity to excitation light polarisation is ubiquitously present in the imaging data even if it is not specifically desired by the experiment and needs to be either accounted for or eliminated (Pulin et al., 2022).

8. Imaging in deep tissues and living organisms

Imaging GPCR signalling in live organisms is the ultimate setting for understanding underlying physiological processes. However, imaging thick tissue samples or whole animals present a unique set of challenges. Major issues include the inability of visible light to penetrate deep into the sample due to high scattering, the strong autofluorescence of many tissue components, complications with targeted ligand delivery, photodamage, and oxidative stress of the samples. These issues are addressed by several techniques including bioluminescence imaging, 2P microscopy, optogenetic signalling regulation, and LSFM (Figures 2 and 3, Supporting Table 1).

8.1. Bioluminescence imaging

Many tissues contain molecules with strong autofluorescence; therefore, the use of fluorescence excitation with visible light in such samples is complicated. Bioluminescence imaging allows background-free image acquisition since no excitation light is required (Figure 2m). Bioluminescence is produced by the chemical transformation of a substrate by the luciferase enzyme. The absence of a background signal allows for very long exposure times (minutes) to detect even very weak luminescence intensities. However, this is also a major drawback of bioluminescence imaging because such long exposure times are often required for image acquisition, limiting its applicability for the study of dynamic processes. A particularly promising approach is bioluminescence complementation, which is based on the reconstruction of a complete functional luciferase by binding its two split pieces together (Remy & Michnick, 2006). Bioluminescence complementation has been used in live mice to detect the interactions of β-arrestin2 with β2AR (Takakura et al., 2012) and sphingosine-1-phosphate receptor 1 (Kono et al., 2017) and visualise β2AR activation by ligands in breast cancer cells (Alcobia et al., 2018). Bioluminescence has been used to report Gs activation in live mice using the cAMP response element (Dressler et al., 2014). Bioluminescence imaging is also compatible with high throughput screening reporting GPCR interactions with β-arrestins in multi-well plates (Hattori & Ozawa, 2015).

8.2 2P fluorescence microscopy

Another approach to reducing sample autofluorescence and excitation light scatter is 2P fluorescence microscopy. 2P microscopy relies on the excitation of fluorophores with two photons of infrared light instead of a single visible light photon (Figure 2n) (Denk, 1994). The main advantages of 2P microscopy include its penetration depth allowing imaging a few millimetres deep inside living tissue, inherent sectioning ability due to fluorescence excitation only in a small focal volume (2 nanolitres), and a considerable reduction of autofluorescence. The limitations of 2P microscopy include the requirement for an expensive pulsed infrared laser light source and a low efficiency of excitation and hence a low intensity of emitted fluorescence. In GPCR-related research, 2P microscopy was used, for example, for the imaging of Gi-dependent neutrophil accumulation in response to *S. aureus*infection in live mice (Liese et al., 2013). 2P microscopy has been combined with other imaging modalities, such as FRET-FLIM for the imaging of protein kinase A activation by GPCRs in brain slices (Chen et al., 2014) or STED for the intravital imaging of glucagon-like peptide-1 receptor signalling (Ast et al., 2020). Several existing photoswitchable ligands for 2P microscopy can be

used for the regulation of dopamine (Araya et al., 2013) and metabotropic glutamine receptor (Carroll et al., 2015) activity in the brain.

8.3 Light sheet fluorescence microscopy

LSFM or selective plane illumination microscopy (SPIM) is a powerful tool for imaging long-term processes in large samples with minimal photodamage to the sample (Figure 2o) (Voie et al., 1993). LSFM uses a light sheet of laser light to illuminate a thin sample section perpendicular to the observation direction. Therefore, LSFM minimises the duration of illumination for individual parts of the sample, reduces phototoxicity, and enables long-term observations with good axial resolution. LSFM has been used for imaging downstream signalling from activated adrenergic receptors in acute brain slices (Pham et al., 2020).

9. Imaging of endogenous signalling systems

The reliable study of physiological processes requires minimal perturbations of endogenous systems. However, achieving this goal remains a challenging task. Major (a thousand-fold (Cho et al., 2022)) differences in the expression of GPCR cascade components make it very difficult to find a "one-size-fits-all" microscopy technique for the study of endogenous systems. However, the advent of versatile CRISPR-based gene editing has initiated a new era of GPCR studies using endogenous protein expression levels. CRISPR technology allows the modification of endogenously expressed proteins with genetically encoded fluorescent and luminescent probes. The most prominent examples of CRISPR applications in GPCR research include the Open Cell project, which has produced and made available an outstanding amount of imaging data on the localisation and expression levels of endogenous signalling components (Cho et al., 2022), and the removal of selected endogenous G protein subunits (Alvarez-Curto et al., 2016) and GRKs (Drube et al., 2022) from HEK293 cells which has enabled the study of signalling in a background-free setting. Careful choice of the reporting optical probes is required for the study of endogenous systems. Small (subnanometer) probes, with high labelling specificity that do not alter the signalling properties of the studied system, can be delivered to the site of labelling, and are compatible with *in vivo* imaging will be most useful for the study of endogenous systems. Examples of such probes include fluorescent ligands (Rosier et al., 2021), ligand-directed fluorescent labelling probes (Stoddart et al., 2020), or probes for direct protein labelling with click chemistry of unnatural amino acids (Arsic et al., 2022; Mihaila et al., 2022). The development of CRISPR in combination with genetic code expansion and optimised optical probes will provide major insights into endogenous signalling systems in the future.

Label-free techniques are a separate group of methods suitable for the study of endogenous systems. For example, surface plasmon resonance and dynamic mass redistribution (Figure 2l) have been used to establish the role of PDZ-ligands in GPCR signalling (Camp et al., 2016) and detect angiotensin 1 receptor activity in a label-free fashion (Drube et al., 2022). Atomic force microscopy (AFM), which does not truly belong among optical approaches, finds its uses in GPCR research. AFM scans the sample surface with a fine probe to determine its 3D landscape and the interaction forces between molecules. It has been used to determine the ligand-binding free energy of PAR1 (Alsteens et al., 2015) and detect GPCR oligomerisation through receptor unfolding (Dague et al., 2022). Further development of label-free technologies will expand their areas of application to provide holistic insights into endogenous cell signalling.

10. Labelling strategies and biosensors

Over the years a variety of labelling strategies and biosensors suitable for imaging GPCR signalling have been developed (Figure 4). Finding the optimal probe or their combination is a cornerstone of any imaging project because different probes have their advantages, limitations, and areas of application. The most common labelling strategies applied in GPCR research include the use of fluorescent ligands, FPs, direct and ligand-directed labelling, self-labelling systems, protein conjugation systems, quantum dots, and nanodiamonds. Existing biosensors are used to detect a multitude of signalling processes, including ligand binding (Rosier et al., 2021), signalling cascade activation (Maziarz et al., 2020), GPCR conformation changes (Banerjee et al., 2022), receptor heteromerization (Sleno et al., 2017), interactions between signalling proteins (Olsen et al., 2020), the release of neurotransmitters (Patriarchi et al., 2018; Sun et al., 2018), signalling in different

subcellular compartments (Irannejad et al., 2013), the production of second messengers and their spatial localisation (Anton et al., 2022), and many others.

10.1 Labelling approaches

Fluorescent ligands label GPCRs and simultaneously ensure a defined receptor conformation (Figure 4a). A unique advantage of fluorescent ligands is the elimination of direct covalent labelling of signalling system components. Therefore, fluorescent ligands are suitable for imaging endogenous systems and the dynamics of GPCR activation and activity regulation. Fluorescent GPCR ligands span a range of applications from SPT (Isbilir et al., 2020) to high-throughput screening (Tahk et al., 2023) and are promising tools for the study of endogenous systems (Barbazan et al., 2022). However, this comes with the limitation that fluorescent ligands can only visualise the proteins to which they bind.

The ligand-directed fluorescent labelling approach relies on a GPCR ligand as a carrier for a fluorophore that is covalently attached to the receptor upon ligand binding and remains attached after ligand dissociation (Figure 4b) (Stoddart et al., 2020). This approach allows the labelling of endogenous GPCRs while maintaining their ability to be activated and inactivated. Ligand-directed labelling can be used in live animals (Arttamangkul et al., 2019). Ligand-directed labelling requires the presence of suitable amino acids for labelling on the receptor surface and is often limited by the existence of a suitable ligand conjugated with the appropriate fluorophore. This technique can be combined with other labelling schemes to create a RET pair for studies of GPCR signalling with FRET techniques.

Direct labelling is used mainly for *in vitro* experiments with applications such as smFRET and FCS and is usually based on the labelling of protein cysteines or introducing unnatural amino acids with fluorescent dyes (Figure 4c) (Mihaila et al., 2022). This approach ensures the smallest possible label size and allows for colour multiplexing. However, it has very limited compatibility with the imaging of live samples and often suffers from non-specific labelling and different labelling efficiencies causing artefacts in multicolour experiments.

FPs are workhorses of optical microscopy that are compatible with multiple imaging techniques and serve a variety of purposes (Figure 4f). FPs can be split into parts and restored using bimolecular fluorescence complementation, undergo photoswitching and photoactivation required for SMLM techniques, serve as RET donors and acceptors, can be used with 2P excitation in deep tissues, or even assist GPCR purification (reviewed in (Kim et al., 2022; Ravotto et al., 2020). The limitations of using FPs include their relatively large size, which limits localisation precision for SRN techniques and often interferes with signalling, irreversibility of labelling, and fast bleaching.

Self-labelling systems , including SNAP-tag, CLIP-tag (Gautier et al., 2008), and HaloTag (Los et al., 2008), are based on a genetically encoded modification of the molecule of interest with an enzyme which catalyses its covalent labelling with a reactive fluorescent dye (Figure 4d). These versatile systems enable specific labelling with the possibility of multiplexing and flexible experimental design, due to the existence of a variety of suitable dyes of different colours with distinct membrane permeability, some of which can be photoactivated. Self-labelling systems allow the study of multiple processes using the same labelled signalling protein. Common applications of these systems include SPT which takes advantage of the labelling specificity, photostability, and brightness of available dyes (Asher et al., 2021; Sungkaworn et al., 2017). The limitations of self-labelling systems involve mainly their large size, which is comparable to that of an FP.

Protein conjugation systems , including ALFA-tag (Gotzke et al., 2019), SpyTag (Alam et al., 2019) and binder-tag (Liu et al., 2021), rely on the target protein modification with a small peptide (7-14 amino acids) and binding of the universal fluorescently labelled small antibody-like protein. Irreversible (ALFA-tag, SpyTag) or reversible (binder-tag) conjugation allows imaging of signalling with only small direct modifications of the studied protein (Figure 4g). Moreover, in the case of binder-tag technology, the binding itself depends on protein conformation and is indicative of signalling activity. Protein conjugation systems are very promising but include relatively large labels and require delivery of exogenous fluorescently labelled proteins for labelling the experimental system.

Quantum dots and nanodiamonds provide an alternative to classical fluorescence dyes due to their outstanding brightness and photostability (Figure 4e) (Sotoma et al., 2016) and can be combined with self-labelling systems (Komatsuzaki et al., 2015). They are optically superior to fluorescent dyes but suffer from their large size (2-50 nm) and complicated intracellular delivery for applications in SPT and emerging SRN techniques.

10.2 Biosensors

An extensive toolbox of biosensors for GPCR signalling is available to researchers (Kim et al., 2022; Olsen & English, 2023). Here, we showcase only a few important examples (Figure 4h-l). Nanobodies initially developed as a structural tool have become widely used in the imaging of GPCR signalling (Figure 4h). These small single-chain antibodies, originally derived from camelids, selectively bind to proteins in particular conformations or protein complexes in particular compositions (Rasmussen et al., 2011). A closely related group of sensors are miniG proteins (Nehme et al., 2017; Wan et al., 2018), nanobody-like proteins based on the GTPase domain of Gα subunits, which bind to activated GPCRs and mimic G protein binding (Figure 4i). Nanobodies and miniG proteins have been used for the detection and regulation of many GPCR signalling aspects (Gil et al., 2020; Gormal et al., 2020) and the determination of subcellular localisation of signalling molecules (Irannejad et al., 2013). Another notable group of GPCR-based biosensors include dopamine receptors with inserted circularly permuted FPs which allow the quantitation of neurotransmitter release at a wide range of concentrations in live cells and animals (Figure 4l) (Patriarchi et al., 2018; Sun et al., 2018). Intracellularly recently developed FRET-based GPCR nanoruler sensors measure the concentration of second messengers, cAMP in particular (Anton et al., 2022), for the accurate detection of distances at which signalling effects propagate inside cells (Figure 4j). Multiple existing optogenetic GPCR-based sensors activate (Airan et al., 2009) and regulate (Lockyer et al., 2023) GPCR signalling using light pulses instead of chemical compounds (Figure 4k). Photomanipulation can also be used to control GPCR localisation and induce receptor confinement (Sanchez et al., 2021). BRET sensor systems such as BERKY and TRUPATH allow sensitive measurements of specific GPCR and G protein activity (Maziarz et al., 2020; Olsen et al., 2020). These and many other sensors provide an extensive set of tools for the detection and targeted regulation of GPCR signalling.

11. Conclusions and future perspectives

Many microscopy and spectroscopy approaches are available for studies of GPCR signalling at multiple levels from individual molecules to whole organisms. Future developments in microscopy techniques, particularly SRN approaches (MINFLUX, RASTMIN, MINSTED, and RESI), will bridge the gap between optical microscopy and structural biology. More extensive use of the traditionally "*in vitro* " techniques with high temporal resolution, such as smFRET and nanosecond FCS, in living cells and organisms will enable dynamic signalling studies in a more complex native setting. The utilisation of previously underappreciated optical phenomena and the development of label-free technologies will allow the extraction of additional information without increasing the complexity of experimental systems. These advances will allow the determination of structural and dynamic properties of GPCR signalling in conditions closer to natural than previously possible.

The development of new labelling approaches and small specific labels with enhanced optical properties will allow use of the full potential of new microscopy techniques. The use of CRISPR gene editing along with genetic code modification and unnatural amino acids will allow the targeted labelling of endogenous proteins with minimal perturbation to endogenous systems. The development of new small and specific labels, including high-affinity fluorescent ligands, dyes suitable for click-chemistry with unnatural amino acids, and ultimately fluorescent amino acids, will enable the precise detection of conformational changes, the localisation of cascade members, and their interactions with the environment and other proteins at the level of individual molecules in endogenous systems.

Machine learning and artificial intelligence (AI) are already revolutionising many fields of science, including microscopy, and signalling studies. Machine learning AI will enable a high degree of automatization in image

acquisition and analysis, as well as the extraction of previously unavailable information from imaging data. It will likely lead to the development of new imaging approaches and revolutionise data analysis. The future conversion of complex microscopy approaches such as SPT into high-throughput techniques will qualitatively change the amount and accuracy of the information we can obtain to understand the properties of the GPCR signalling cascade.

In summary, future developments in microscopy, spectroscopy techniques and data analysis algorithms will unify the data obtained at multiple scales and allow researchers to understand endogenous signalling in unpreceded detail and precisely modulate its function.

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Figure legends

Figure 1. Spatiotemporal characteristics of the GPCR signalling cascade. (a) Temporal dynamics of individual signalling stages. Individual signalling stages range in their duration from nanoseconds (or even femtoseconds if initial steps of rhodopsin signalling are considered) to tens of minutes. (b) Spatial properties of signalling steps. Spatial properties of the signalling cascade range from Angstrom movements, which accompany conformational changes, to tens of centimetres, which characterise neuronal signalling and hormonal distribution.

Figure 2. Microscopy and spectroscopy approaches for the study of GPCR signalling. (a) STED is an SRN technique that uses a donut beam to quench all fluorophores outside a small central spot. (b) PALM and STORM use weak light intensity combined with photoactivation of fluorescent proteins or blinking of dyes to acquire resolution enhancement by imaging individual molecules. (c) DNA-PAINT uses transient binding of fluorescent oligonucleotides to reproduce the blinking behaviour required for image reconstruction from multiple frames with individual fluorescent molecules. (d) MINFLUX scans the area looking for a central minimum of fluorophore excitation corresponding to its precise position. (e) SIM projects patterns of light on a sample allowing image reconstruction with improved resolution. (f) Expansion microscopy is based on the creation of a swellable polymer network with a sample and expansion of it to enable enhanced resolution of fine sample details. (g) SmFRET allows accurate measurements of protein conformational changes and protein-protein interactions at the single-molecule level and high temporal resolution. (h) FLIM allows accurate detection of fluorescence lifetimes to filter out background signals and achieve intensity-independent reproducible measurements. (i) SPT acquires images of sparse samples and uses image processing to accurately determine the subpixel localization of individual molecules and perform their tracking with high temporal resolution. (j) FCS utilises fluorescence fluctuations to obtain information on sample dynamics with a very high temporal resolution. (k) Polarisation microscopy gains insights into protein conformation and interactions by taking advantage of the fact that light absorption in many fluorophores in constrained systems, such as cell membranes, depends on light polarisation. (l) DMR is a label-free technique that uses waveguides to detect minute changes in the distribution of cellular mass that accompany signal transduction. (m) Bioluminescence imaging uses a chemical reaction catalysed by luciferases, which is accompanied by the emission of light, to detect signalling without using excitation light and with a minimal nonspecific background. (n) 2P microscopy allows deep tissue imaging with low scatter and low autofluorescence by using two photons of infrared light instead of a single visible light photon to excite fluorescent molecules. (o) LSFM uses a sheet of light to fast scan large sample areas with minimal photodamage.

Figure 3. Spatiotemporal resolution of optical microscopy and spectroscopy techniques. (a) Temporal resolution limits of microscopy and spectroscopy techniques. (b) Spatial resolution of imaging techniques.

Figure 4. GPCR labelling strategies and examples of biosensors. (a) Fluorescent ligands simultaneously modulate GPCR activity and fluorescently label the receptors. (b) Ligand-directed labelling is based on receptor binding by a ligand bearing a fluorescent label that becomes covalently attached to the receptor. Afterwards, the ligand can be washed out, leaving the labelled GPCR. (c) Direct fluorescent labelling is based on the direct binding of a fluorescent dye to protein cysteines or unnatural amino acids. (d) Self-labelling systems (e.g., SNAP-tag, HaloTag) autocatalyse their specific labelling with fluorescent dyes. (e) Quantum dots and nanodiamonds can label specific reactive groups attached to proteins of interest and provide extraordinary brightness and photostability. (f) Fluorescent proteins are a major group of commonly used genetically encoded labels. Photoswitchable and photoactivatable fluorescent proteins are extensively used in SRN techniques. (g) Protein conjugation labelling systems (e.g., ALFA-tag, SpyTag) specifically bind a small amino acid sequence in the protein of interest. (h) Nanobodies are small single-chain antibodies that bind GPCRs in a specific conformation. (i) MiniG proteins are nanobody-like surrogates based on the GTPase domain of Gα subunits that irreversibly bind to activated GPCRs. (j) GPCR-based cAMP nanorulers allow for the detection of cAMP production with high spatial precision. (k) OptoGPCRs contain extracellular parts of rhodopsin and intracellular parts of other GPCRs so that they can be activated by light and produce a signal from those GPCRs intracellularly. (l) Circularly permuted fluorescent protein-based (cpFP) neurotransmitter sensors allow the quantitative detection of neurotransmitter concentration in the vicinity of a cell using GFP fluorophore sensitivity to the environment.

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