UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE
FACULTY OF SCIENCE

Studies of membrane protein structure and function using polarization microscopy

Ph.D. THESIS

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Annotation

Membrane proteins are notoriously hard to study because of their requirement for a lipid membrane for function. We have developed the technique of two-photon polarization microscopy (2PPM) which takes advantage of protein membrane localization and yields insights into membrane protein structure and function in live cells and organisms. 2PPM utilizes linear dichroism of a fluorescent label for detection of conformational changes and protein-protein interactions in membrane proteins in live cells. We have applied 2PPM to studies of the G protein signal transduction. We show that 2PPM can be utilized for observing interactions between G protein subunits, and detection of G protein activation in live cells, in real time. Using 2PPM, along with other techniques, we demonstrate that Gi/o protein heterotrimers dissociate into free Gαi/o and Gβγ subunits upon activation, and these dissociated subunits represent the major active form of G proteins. Next, we show that 2PPM can be used for detection of interactions between G protein-coupled receptors and G proteins. Our 2PPM results indicate that Gi/o proteins do not precouple to their cognate GPCRs in the inactive state. Our results demonstrate that 2PPM is a promising novel technique for studies of membrane proteins, yielding information both on their structure and function. 2PPM requires only a single fluorescent label and can utilize many existing constructs. 2PPM allows studies of membrane proteins in conditions closer to natural than previously possible.

Declaration [in Czech]

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Alexey Bondar

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The thesis is based on the following publications:

I. Josef Lazar, **Alexey Bondar**, Stepan Timr and Stuart Firestein. **(2011)** Two-photon polarization microscopy reveals protein structure and function. *Nature Methods*, 8, 684-690. (IF = 23.6)

II. **Alexey Bondar**, Josef Lazar. **(2013)** Dissociated $G\alpha_{GTP}$ and $G\beta\gamma$ protein subunits are the major activated form of heterotrimeric Gi/o proteins. *Journal of Biological Chemistry*, 289, 1271-1281. (IF = 4.7)

III. **Alexey Bondar**, Josef Lazar Gi/o proteins do not precouple to members of the Class A and C of G protein-coupled receptors. *Manuscript*.

List of publications not included in the thesis:

IV. Stepan Timr, **Alexey Bondar**, Lukasz Cwiklik, Martin Stefl, Martin Hof, Mario Vazdar, Josef Lazar and Pavel Jungwirth **(2013)** Accurate Determination of the Orientational Distribution of a Fluorescent Molecule in a Phospholipid Membrane, *Journal of Physical Chemistry B*, 118, 855-863.

V. Zhou Han, Lei Jin, Fuyi Chen, Joseph J. Loturco, Lawrence B. Cohen, **Alexey Bondar**, Josef Lazar and Vincent A. Pieribone Modifications of the genetically encoded fluorescent protein voltage probe ArcLight; mechanism of its voltage sensitivity. Submitted to *PLoS One*.

VI. **Alexey Bondar**, Stepan Timr, Tomas Ryba, Frantisek Batysta, Milos Zelezny, Josef Lazar Functional comparison of one-photon and two-photon polarization microscopy, *Manuscript.*

Letter of declaration

Prohlašuji, že se Alexey Bondar podílel na společných publikacích přibližně v níže uvedeném rozsahu.

I. Josef Lazar, **Alexey Bondar**, Stepan Timr and Stuart Firestein. **(2011)** Two-photon polarization microscopy reveals protein structure and function. *Nature Methods*, 8, 684-690.
AB performed microscopy experiments, prepared constructs, analyzed data, and devised experimental strategies

II. **Alexey Bondar**, Josef Lazar. **(2013)** Dissociated $G\alpha_{GTP}$ and $G\beta\gamma$ protein subunits are the major activated form of heterotrimeric Gi/o proteins. *Journal of Biological Chemistry*, 289, 1271-1281.
AB conceived the idea (jointly with *JL*), devised experimental strategies, conducted experiments, analyzed the data (jointly with *JL*) and wrote the manuscript (jointly with *JL*)

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Josef Lazar, Ph.D.

Dedicated to my beloved wife Kate and son Arkadij

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LIST OF ABBREVIATIONS

1P – One-Photon
2P – Two-Photon
2PPM – Two-Photon Polarization Microscopy
α2A-AR – α2A-Adrenergic Receptor
AC – Adenylyl Cyclase
APB – Donor Recovery after Acceptor Photobleaching
ATP – Adenosine Triphosphate
BRET – Bioluminescence Resonance Energy Transfer
cAMP - 3'-5'-cyclic Adenosine Monophosphate
CCD – Charge-Coupled Device
CFP – Cyan Fluorescent Protein
cleGFP – C-terminally lipidated enhanced Green Fluorescent Protein
cpV – circularly permuted Venus
dleGFP – dually lipidated enhanced Green Fluorescent Protein
DMEM – Dulbecco Modified Eagle Medium
eCFP – enhanced Cyan Fluorescent Protein
eGFP – enhanced Green Fluorescent Protein
eYFP - enhanced Yellow Fluorescent Protein
FP – Fluorescent Protein
FLIM – Fluorecence Lifetime Imaging Microscopy
FRAP – Fluorescence Recovery After Photobleaching
FRET – Förster Resonance Energy Transfer
GABA – γ-Aminobutyric Acid
$GABA_B$ - γ-Aminobutyric Acid Receptor Type B
GDP – Guanosine diphosphate
GECI – Genetically-encoded Calcium Indicator
GFP – Green Fluorescent Protein
GIRK – G protein-regulated Inward Rectifying Potassium Channel
GPCR – G Protein-Coupled Receptor
GRK – G Protein-Coupled Receptor kinase
GRK3ct – C-terminal fragment of G Protein-Coupled Receptor Kinase 3
GTP – Guanosine Triphosphate

HBSS – Hank's Balanced Salt Solution
HEK293 – Human Embryonic Kidney cells 293
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHBSS – HEPES buffered Hank's Balanced Salt Solution
ileGFP – internally lipidated enhanced Green Fluorescent Protein
LD – Linear Dichroism
NA – Numerical Aperture
NE – Norepinephrine
PBS – Phophate Buffered Saline
PKA – Protein kinase A
PKCγ – Protein kinase Cγ
PTX – Pertussis toxin
RPM – Rapid Polarization Modulator
TDM – Transition Dipole Moment
TIRF – Total Internal Reflection Fluorescence Microscopy
wtGFP – wild-type Green Fluorescent Protein
YFP – Yellow Fluorescent Protein

1. Introduction

“Seeing is believing”

Our eyes provide us with more than 80% of total information we get from the outside world (1). Therefore, it is in human nature to trust in what we see. It is easy to see macroscopic objects surrounding us but not nearly as easy to see other, much smaller objects. The help of special devices, like a magnifying glass, is needed to see them. Human intrinsic interest in what the human body is made of and how it works required finding a way to perceive it in the finest detail. Since we trust our eyes the most, it is natural that the best way to find out how our body works is to see its composing elements with the greatest possible magnification. We, therefore, needed a way to see what cannot be seen with an unarmed eye. And that is why microscopy, which is using microscopes to view samples and objects that cannot be seen with the unaided eye, was invented. While early microscopy studies mostly provided information only about appearance of the studied object, there was a tremendous urge to see how things work in living organisms and what makes them work like that. Years of technology development finally made it possible to see processes occurring in the smallest living entities – live cells, on the molecular level.

A cell is the unit form of the living matter. It possesses all structural and functional properties required for independent self-replication. Therefore, studies of live cells provide us with crucial information about basic principles of living matter existence. However, elucidation of structure of live cell components and their functional activity is a challenging task.

Protein molecules carry out majority of functions in live cells. They can serve as building blocks, receptors or enzymes, and, therefore, are outstandingly important. Membrane-bound proteins are particularly interesting because membranes separate cells from extracellular environment and form all intracellular organelles. However, despite a plethora of functions carried out by membrane proteins their studies have always been difficult due to the requirement for a lipid membrane for their proper folding and functional activity. In our research we aimed to take advantage of membrane protein localization for studies of their structure and function in live cells.

1.1. Structure and function of membrane proteins

Membrane proteins form one of three major protein classes along with fibrous proteins and globular proteins. Membrane proteins represent a large and diverse group of proteins which are attached to cellular membranes and carry out a wide range of functions. Membrane proteins can be divided into subcategories according to their functional activity:

1. Structural proteins ensure cell membrane integrity and cellular stability.
2. Enzymes carry out enzymatic reactions at cellular membranes.
3. Cell adhesion proteins are responsible for recognition of other cells.
4. Receptors transduce signals from extracellular environment.
5. Transport proteins carry out transport of various chemical substances across the cell membranes and take part in homeostasis maintenance.
6. Ion channels regulate ion flux across cellular membrane.

Cellular metabolism and communication heavily rely on membrane proteins. More than 30% on all proteins interact with membranes at some stage of their functional activity (2). Therefore, studies of membrane proteins are crucial for understanding of mechanisms of cellular operation.

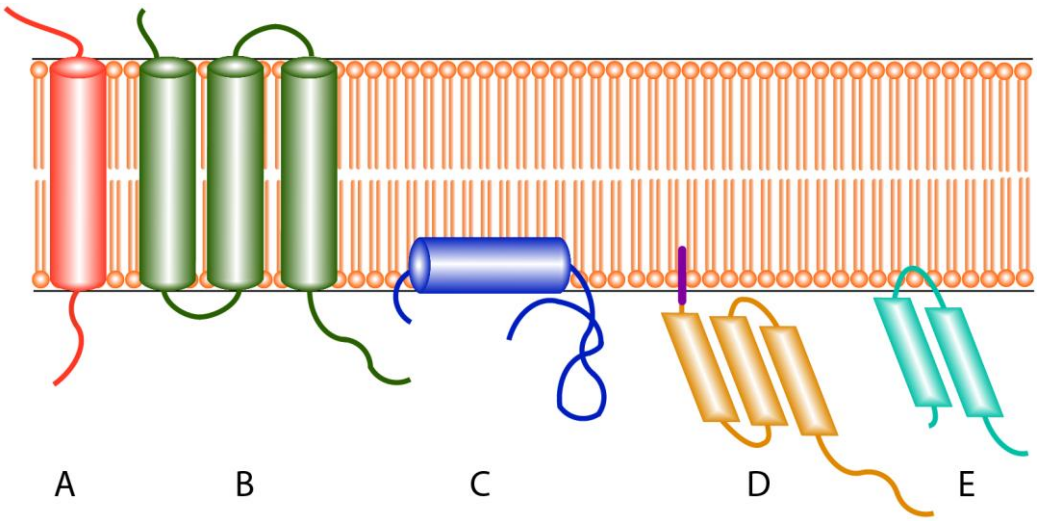

Figure 1. Examples of integral and peripheral membrane proteins. Monotopic (A) and polytopic (B) integral membrane proteins. Peripheral membrane proteins can be differently attached to the membrane through an α-helix parallel to the membrane (C), covalently attached lipid tag (D), a loop (E) or electrostatic interaction.

Membrane proteins can be divided into integral and peripheral membrane proteins according to their localization in the membrane. Integral proteins penetrate the membrane and are permanently located in it. In experiments integral membrane proteins can only be separated from the membrane using detergents. Peripheral membrane proteins are often transiently located in the membrane and can detach from it in course of their functional activity. Integral and peripheral membrane proteins can be classified according to their attachment to cell membranes (Figure 1).

Requirement of the lipid membrane for proper functional activity of membrane proteins makes them particularly hard to study both *in vitro* and *in vivo*. Modern biochemistry offers a limited number of methods for studies of proteins in lipid environments. Lipophilic properties of membrane proteins impede their purification. Proper folding and conformation of membrane proteins are hard to achieve and preserve without the membrane part. Addition of artificial membranes seldom provides environment equivalent to cellular membranes; therefore, reconstitution of proper membrane protein activity is a complicated and tedious process. Studies of membrane proteins in live cells are preferred to *in vitro* studies, but they also face a multitude of problems. Fluorescent dyes used for membrane protein labeling in live cells have limited utilization in live animals and often show significant non-specific labeling. Utilization of genetically-encoded probes, in particular based on fluorescent proteins (FPs), is advantageous over using the dyes because it allows ensuring labeling specificity, controlling the expression levels and working in animal models. However, labeling of membrane proteins with FP has its own drawbacks. Membrane proteins are very sensitive to modification of their structure by FP insertion and offer a smaller choice of sites for FP insertion compared to cytoplasmic proteins. Multiple functional tests are required to ensure that the target protein retains its function and the mechanism of this function remains unchanged. Therefore, experimental techniques which allow studying membrane proteins in conditions as close to natural as possible are of high demand.

Membrane proteins are outstandingly important not only for biological science but also for medicine and pharmacology because more than 50% of modern drugs target membrane proteins. Despite several Nobel prizes already

awarded for studies of membrane proteins and recent advances in membrane protein studies there is still no complete picture of structure and functional activity of these proteins. Therefore, we developed the technique of two-photon polarization microscopy (2PPM) in order to facilitate research of membrane proteins and answer some the most difficult long-standing questions in this field.

1.2. Aims of the research:

The principal goal of our work was to develop a microscopy technique for studies of membrane proteins in live cells and apply this technique to address interesting and important scientific questions.

Specific aims of the research were to:

1. Develop a microscopy technique for studies of membrane proteins in live cells, which would take advantage of their membrane localization
    a. Develop theoretical background and practical implementation of the technique
    b. Apply this technique for detection of protein-protein interactions in live cells
    c. Utilize this technique for determination of membrane protein conformational changes in live cells
    d. Obtain structural information about membrane proteins with this technique
2. Determine whether the activated form of Gi/o proteins is a re-arranged heterotrimer or free $G\alpha i/o_{GTP}$ and $G\beta\gamma$ subunits.
    a. Determine whether heterotrimeric Gi/o proteins dissociate upon activation in live cells
    b. Determine the extent of Gi/o heterotrimer dissociation upon activation
    c. Ascertain significance of Gi/o heterotrimer dissociation for interaction with downstream effectors
3. Determine whether Gi/o proteins physically interact (precouple) with their cognate GPCRs in the inactive state
    a. Determine whether Gαi/o subunits physically interact with GPCRs in the inactive state

b. Ascertain whether Gβγ dimers facilitate interaction between inactive Gα subunits and GPCRs
c. Determine whether GPCRs of different classes exhibit distinct modes of interaction with Gi/o proteins

1.3. Introduction to microscopy

Microscopy is a general term for experimental techniques used for visualization of objects and details which are too small to be seen by the unaided eye. All methods of microscopy rely on utilization of microscopes – special devices for obtaining magnified images of the objects of interest.

Three main kinds of microscopy exist:

1. optical microscopy,
2. electron microscopy,
3. scanning probe microscopy.

Optical microscopy utilizes visible, ultraviolet (UV) or infrared (IR) light for detection and magnification of objects. All optical microscopy techniques utilize either scattering or absorption of transmitted light, or detect light emitted by the sample itself (fluorescence microscopy). Optical microscopy is currently the most common and efficient way of studies living samples because they can be studied in their natural environment or in conditions ensuring their survival. Visible light used in optical microscopy (and to lesser extent UV and pulsed IR light) does not seriously harm the samples. Fluorescence microscopy also allows visualizing specific proteins or organelles in living cells thereby allowing studies of cell functional activity at the molecular level. The biggest drawback of optical microscopy techniques is the diffraction limit which restricts the highest achievable resolution to ~200 nm (3). However, recent developments of several superresolution microscopy techniques allow breaking the diffraction limit and potentially eliminating the difference in highest resolution between optical and electron microscopy techniques (4).

Electron microscopy uses a beam of electrons for sample illumination and production of a sample image. Since electrons have much shorter wavelength compared to visible light, electron microscopy is virtually not limited by diffraction. Two main techniques of electron microscopy are transmission electron microscopy and scanning electron microscopy. The best resolution is

achieved by transmission electron microscopy and can be as high as 0.05 nm (5). Electron microscopy techniques cannot be applied to living samples because of high energy of the electron beam and requirement for imaging samples in vacuum. Therefore, electron microscopy can be effectively used for structural studies but has very limited application for studies of functional activity.

Scanning probe microscopy is based on scanning a surface of an object with a tip and registering the shape of the scanned surface. Scanning probe microscopy is not limited by diffraction and allows determination of the sample shape with a resolution dependent only on the volume of interaction between the tip and the sample. Unlike electron microscopy scanning probe microscopy does not require vacuum and can be used for studies of living objects. Many kinds of scanning probe microscopy exist; they can be utilized for studies of material's structure, cell membrane shape or even protein functional activity. However, scanning probe microscopy has certain disadvantages, the biggest of which is its limitation to scanning surfaces and inability to penetrate inside living cells and detect processes occurring there. Also, the effect of the microscope scanning probe on the sample is hard to predict and results may be seriously affected by it.

All three main kinds of microscopy have their areas of application as well as advantages and disadvantages. The most effective approach is combining different techniques together with non-microscopy methods to get the most comprehensive information about the studied process and cross-verify obtained results.

Since we are interested in functional activity of membrane proteins in live cells we utilized the techniques of fluorescence optical microscopy in the present research.

1.4. Fluorescence microscopy

Fluorescence microscopy utilizes emission of light by excitable molecules (fluorescence) for detection of these molecules and studies of their properties.

1.4.1. Fluorescence

Fluorescence is the emission of light by a substance after absorption of light of typically a shorter wavelength. The difference between excitation and emission light wavelengths is called the Stokes shift. This shift allows separating the emitted fluorescence from the excitation light by blocking the latter one completely, which allows detection of only fluorescent objects (6).

Molecules which possess fluorescent properties are called fluorophores. Absorption of a photon (or two photons in two-photon excitation) of light leads to a transition of the fluorophore from the "ground" state to the "excited" state. This transition is a very fast process which happens in femtoseconds (7). There are two ways of return of the fluorophore to the "ground" state: vibrational relaxation and fluorescence emission. The first way does not involve emission of light and absorbed energy is dissipated in the form of heat. The second way is a radiative transition of the fluorophore into the "ground" state. Vibrational relaxation happens on a picosecond timescale while fluorescence emission takes nanoseconds to occur.

Excitation and emission events are usually described and visualized by Jablonski diagrams first developed by Alexander Jablonski is 1930s (8). Fluorophore molecules are typically present in non-excited ground state, which is a singlet state S_0. Absorption of photons leads to a transition of molecule to S_1 or S_2 singlet excited states. Each fluorophore can absorb photons of a range of wavelengths and emit fluorescence in a range of wavelengths as well. These ranges are called excitation and emission spectra. Absorption and emission properties of fluorophores depend on the energy which is needed for transition from S_0 to S_1 or S_2 state and back. Direct return of the fluoropohore from S_1 excited state to S_0 state is accompanied by emission of a photon of fluorescence. If the fluorophore first switches to the triplet state its return to S_0 state is accompanied by longer wavelength phosphorescence. Non-radiative transfer back to the ground state occurs if the energy of the fluorophore transfer from S_1 to S_0 state is dissipated as heat (vibrational relaxation). Jablonski diagram of fluorescence is shown in Figure 2.

Fluorescence is often used in biological studies because it can be well separated from the excitation light and because the object of interest (a

molecule, a membrane, an organelle, or a cell) can be exclusively labeled with a particular fluorophore ensuring specificity of the detected fluorescent signal.

In the course of development of a technique for studies of membrane proteins we utilized three major techniques of fluorescence microscopy: widefield fluorescence microscopy, confocal microscopy and two-photon microscopy. In the following section we will describe these three techniques.

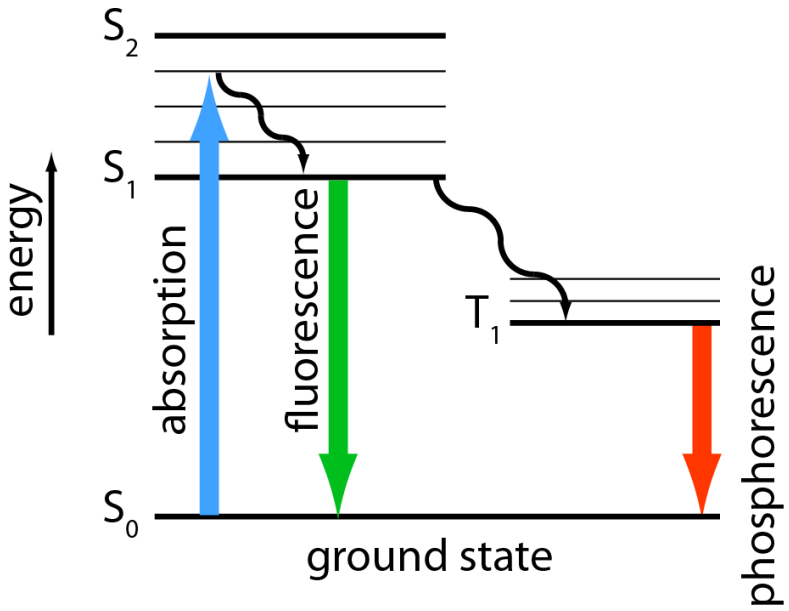

Figure 2. Jablonski diagram. Absorption of a photon of light transfers the fluorophore from its ground state S_0 into one of the excited states S_1, S_2, etc (blue arrow). Radiative transfer of the fluorophore from its excited state back to the ground state is accompanied by fluorescence emission (green arrow). If fluorophore undergoes non-radiative transfer to the triplet state T_1 and then radiative transfer to the ground state emitted phosphorescence has considerably longer wavelength than fluorescence.

1.4.2. Widefield fluorescence microscopy

Widefield fluorescence microscopy is the most commonly used type of fluorescence microscopy with no spatial filtering of fluorescent signal. This technique is easy to implement and does not require sophisticated equipment, tricky alignment and synchronization procedures. In a typical setup (Figure 3), light from a non-collimated light source, which is often an arc lamp, is directed towards a sample (fluorescently labeled or intrinsically fluorescent) through an

excitation filter and an objective lens. Fluorescence is collected through the same objective lens. Emitted fluorescence is separated from the excitation light by a dichroic beamsplitter and an emission filter. A camera (CCD or even a regular photo camera) is commonly used as a detector. Fluorescence from the sample can also be observed by eyes through eyepieces. The main advantages of widefield fluorescence microscopy include its easy implementation, imaging the whole field of view at once and possibility to see the sample with one's eyes. Widefield fluorescence microscopy is a useful technique for basic or preliminary experiments. However, this technique possesses serious disadvantages which preclude its effective use in many experimental systems. The main disadvantage of widefield microscopy is its low contrast due to the absence of spatial signal filtering in z-axis. Fluorescent signal from the focal plane is always contaminated with fluorescence emitted from above and below of the focal plane. Widefield microscopy cannot be used for deep tissue imaging or in highly scattering samples due to strong scattering of visible light used in this technique. Confocal and 2P microscopy techniques were developed to overcome drawbacks and limitations of widefield fluorescence microscopy.

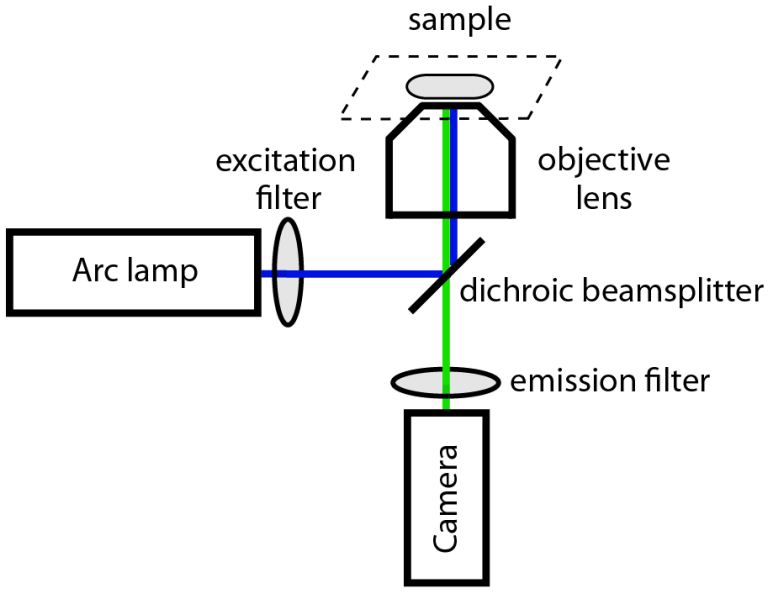

Figure 3. Widefield fluorescence microscopy setup. Non-collimated light from a light source (usually an Arc lamp or an LED) passes through an excitation filter and is reflected by a dichroic beamsplitter through an objective lens onto a sample. Emitted fluorescence is collected through the same objective lens, passes through the dichroic beamsplitter and emission filter to the detector.

1.4.3. Confocal microscopy

Confocal microscopy is a technique which utilizes point illumination and spatial pinholes to physically prevent emitted out-of-focus fluorescence from reaching the detector. Three main kinds of confocal microscopy exist nowadays:

1) laser scanning confocal microscopy (Figure 4),
2) spinning (Nipkow) disk confocal microscopy,
3) programmable array confocal microscopy.

All three techniques typically use point light sources (lasers) and pinholes for imaging the specimen and detecting its fluorescence. In a typical setup, excitation laser light is reflected by a dichroic beamsplitter onto a sample through an objective lens. Fluorescence emitted by the sample passes through the dichroic beamsplitter and before reaching a detector passes through a pinhole which filters out out-of-focus fluorescence. Depending on the type of confocal microscopy, point detectors (photomultipliers (PMTs) or avalanche photodiodes) or CCD cameras can be used for fluorescence detection. The main advantages of confocal microscopy include shallow depth of field, which gives this technique a sectioning ability, and greatly increased contrast comparing to widefield fluorescence microscopy. Confocal microscopy is widely used in biological laboratories. It can be used either for obtaining high resolution images (laser scanning technique) or fast image acquisition (spinning disk or programmable array techniques). Rate of image acquisition is critical for studies of dynamics of biological processes. The main drawbacks of using confocal microscopy include the hard choice between high image resolution and fast image acquisition and its limited application to imaging of thick or highly scattering samples. Another feature, which is a drawback for us, is generally limited potential for customization of available commercial systems. Therefore, combination of confocal microscopy with other microscopy techniques cannot be easily implemented on the majority of available commercial systems. More open and versatile confocal microscopes would speed up the process of introduction of new microscopy techniques into routine laboratory arsenal.

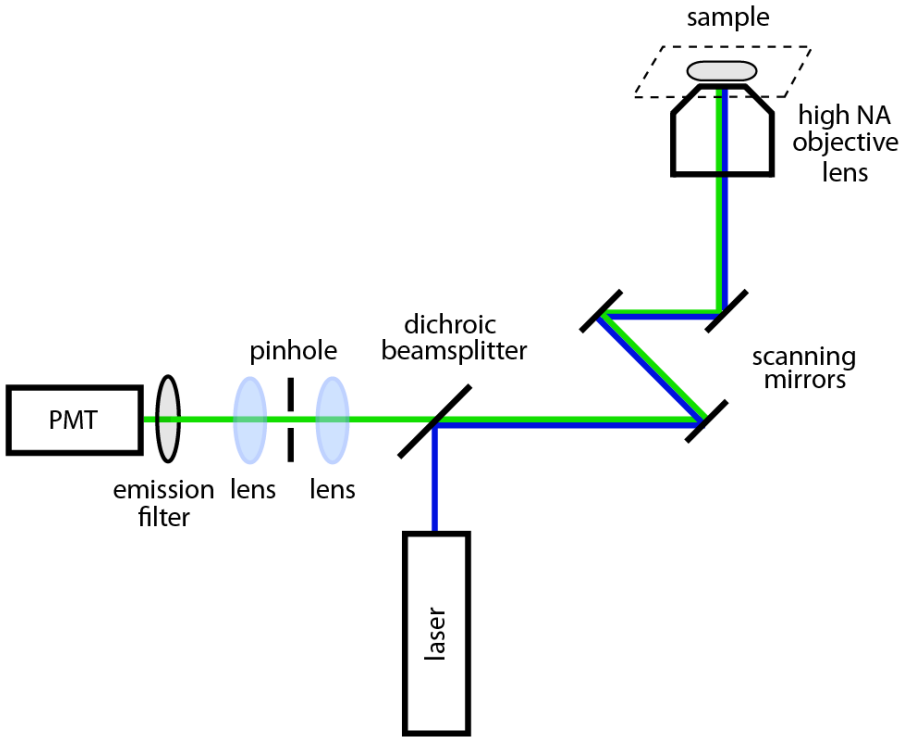

Figure 4. Laser scanning confocal microscopy setup. Light from a laser is reflected by a dichroic beamsplitter into a scanner and then passes through an objective lens onto a sample. Emitted fluorescence is collected by the same objective lens, passes through the scanner, the dichroic beamsplitter and a pinhole, which serves for spatial filtering of fluorescence. After the pinhole, fluorescence passes through an emission filter and reaches a point detector (usually a PMT).

1.4.4. Two-photon microscopy

The concept of two-photon (2P) excitation was originally developed by Marie Goeppert-Mayer in 1931 (9). This concept postulates that two photons of lower energy that together provide comparable energy as one photon required for chromophore excitation can excite the chromophore in one quantum event. 2P excitation is a special case of a broader phenomenon of multiphoton excitation, where the number of exciting photons is ≥2.

After sixty years of further technology development, the technique of 2P microscopy was finally introduced in 1990 by Winfried Denk in the lab of Watt W. Webb at Cornell University (10). This technique utilizes femtosecond pulsed IR lasers because they emit short light pulses of high intensity which is

required for simultaneous absorption of two photons by a single fluorophore and transfer of the fluorophore to the excited state. Figure 5 shows a typical 2P microscopy setup. The setup consists of a light source (femtosecond pulsed IR laser), a scanner, an objective lens, a dichroic beamsplitter, an emission filter and a detector, which is typically a photomultiplier (PMT). 2P microscopy was originally developed as a point scanning technique, in which a sample is scanned point by point and the signal from it is recorded the same way by a sensitive point detector (PMT or an avalanche photodiode).

2P excitation possesses a number of advantages over 1P excitation. 2P microscopy is most commonly used for studies of thick samples such as tissues or even whole living organisms due to the ability of IR light to penetrate deeply into biological samples and excite fluorescence in areas not reachable by visible light. IR light is much less susceptible to scattering than visible light and, therefore, provides much better results in highly scattering samples, such as the brain tissue, where 1P excitation typically fails to excite sufficient number of fluorophores. Another advantage of 2P microscopy is excitation of molecules in only a small (femtoliter size) focal volume (11), which reduces fluorophore excitation outside the focal plane to absolute minimum. This leads to greatly enhanced contrast compared to 1P widefield microscopy and greatly reduced out-of-focus bleaching. Also, utilization of IR light in 2P microscopy usually causes less autofluorescence of the samples than using of visible light.

2P microscopy also possesses several drawbacks and disadvantages. 2P fluorescence is usually weak (11) and requires sensitive detectors. 2P microscopy is a point scanning technique which reduces the rate of data acquisition. Excitation by two photons leads to stronger bleaching of molecules in the focal volume comparing to 1P excitation, although out-of-focus molecules are bleached less. Also, high intensity of light required for 2P excitation leads to strong sample heating. Prolonged constant imaging may even cause boiling of liquid media and “cooking” of the samples. These factors limit temporal resolution of 2P microscopy techniques. However, recent advances in non-scanning 2P microscopy should allow overcoming these limitations (12). It is also worth noting that, although prices have reduced over the last few years, femtosecond pulsed IR lasers remain expensive pieces of equipment.

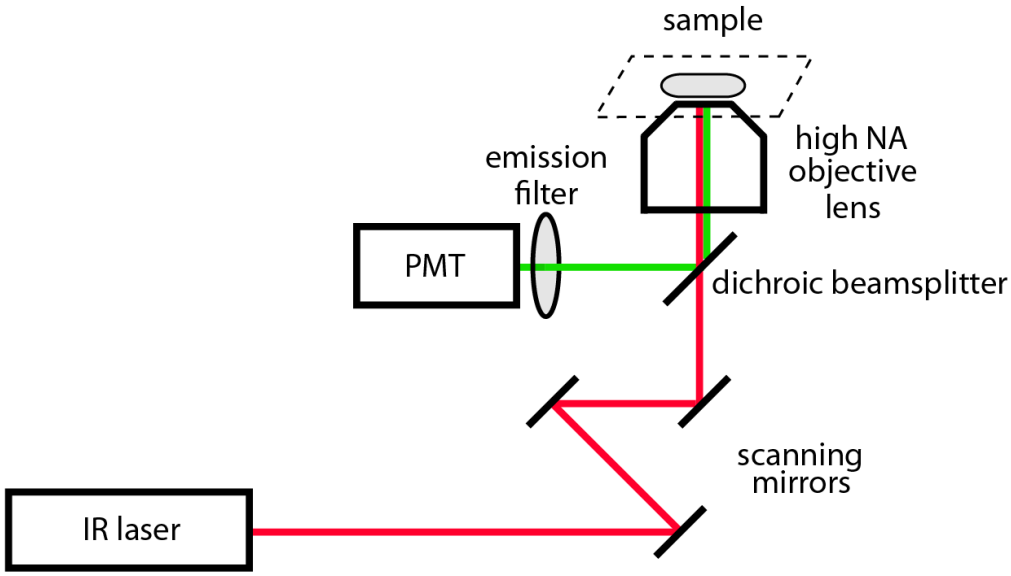

Figure 5. 2P microscopy setup. Tunable femtosecond pulsed IR laser generates pulses of excitation IR light (excitation light beam path is shown in red) which pass through a scanner, a dichroic beamsplitter and a high-NA objective lens onto a sample. Fluorescence emitted from the sample (beam path shown in green) is separated from the excitation light by the dichroic beamsplitter, "purified" by an emission filter and directed into a detector (PMT).

1.5. Fluorescent labels

Three types of fluorescent labels can be utilized for fluorescence microscopy : fluorescent dyes, quantum dots and genetically-encoded fluorescent probes (13).

Fluorescent dyes are small molecules widely used for studies of cellular structure and functional activity. These compounds can be added to the cells or tissues where they chemically interact with certain cellular compounds thereby specifically labeling parts of the cells. The dyes are widely used in biological studies for visualization of cellular organelles or even specific proteins (14). Importantly, dyes are not limited to protein labeling and can also bind DNA, lipids and other intracellular compounds. The main disadvantages of the dyes are a requirement for their external application and a limited ability to control their concentration in cells.

Genetically-encoded fluorescent probes are introduced into the cells in the form of DNA, which is transcribed into RNA and translated into proteins. These probes can be used to label cellular compartments and organelles (by attaching appropriate membrane-targeting or organelle-targeting motifs to the probe) or can be attached to the protein of interest on the DNA level and used to monitor expression levels of that particular protein as well as its localization and function. The most commonly used genetically-encoded probes nowadays are fluorescent proteins derived from the Green fluorescent protein (GFP). These probes allow controlling their intracellular concentration and enable experiments in live animals since no application of external compounds is necessary. They are also less phototoxic in live cells than many of the dyes and even allow genetically-encoded labeling of different cells with different colors (Brainbow mouse (15)). However, genetically-encoded fluorescent probes share some disadvantages of the dyes, such as their potential effect on cellular metabolism or on structure and functional activity of the target protein. Other disadvantages of genetically-encoded fluorescent probes include inability to be utilized in "dead" samples like histological samples, and limited application for labeling non-protein compounds.

1.5.1. Discovery of Green fluorescent protein

GFP was discovered in the jellyfish *Aequorea victoria* by Osamu Shimomura in 1962 (16). However, it took more than 30 years to realize the full potential of GFP. In 1992 Douglas Prasher determined the wild-type GFP (wtGFP) sequence (17) and sent the construct encoding wtGFP to several laboratories in order to find possible applications. The laboratory of Martin Chalfie at Columbia University succeeded in heterologous expression of GFP in *Caenorhabditis elegans* (*C. elegans*) and provided the very first application of GFP (18). A tremendous leap in GFP utilization was made by the Remington group, which determined the structure of S65T GFP mutant, so-called enhanced GFP (eGFP) (19). Since then Roger Tsien has become the leader of mutating GFP into a multitude of spectral variants (Figure 6) (20). Nowadays, thousands of GFP-based sensors of cellular processes exist.

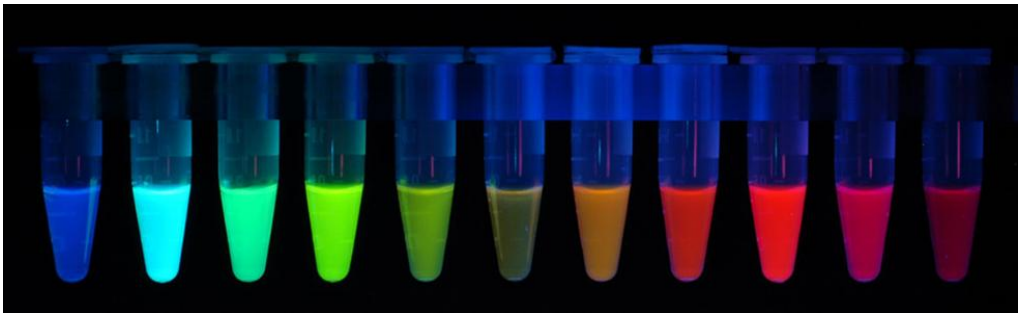

Figure 6. Spectral variants of fluorescent proteins derived from wtGFP (image courtesy of Tsien lab).

1.5.2. Structure of Green fluorescent protein

The GFP molecular structure is a typical β-barrel composed of eleven β-sheets with the fluorophore buried in the middle of the barrel. The structure of GFP is shown in Figure 7. The fluorophore of GFP consists of a 4-(*p*-hydroxybenzylidene)- imidazolidin-5-one structure spontaneously arising by a cyclization and oxidation reaction from three amino acids (Ser-Tyr-Gly) of the polypeptide chain.(21). The GFP fluorophore is a planar system (22). It is worth noting that non-modified Ser-Tyr-Gly tripeptide is not fluorescent by itself and can be found in many proteins not showing any fluorescence properties. Amino acids forming the fluorophore and its surroundings determine the absorption and emission properties of FPs. Mutations in these amino acids lead to changes in excitation and emission spectra as well as in other photochemical properties, such as photostability and quantum yield (20). A whole range of fluorescent proteins (FPs) with excitation and emission spectra spanning from UV to IR is available nowadays.

GFP is widely used as a marker of cellular processes for a number of reasons. GFP folds properly in heterologous expression systems and does not require specific jellyfish factors for adopting its fluorescent form (21). Moreover, GFP is stable and fluorescent both at room temperature and at +37 °C, which is critical for many biological experiments. importantly, GFP does not exhibit any biological activity in the majority of cell types, and often does not affect the structure or functional activity of proteins it is attached to. Therefore, GFP and its derivatives allow specific non-invasive labeling of

cellular proteins or organelles with minimal perturbation of cellular metabolism.

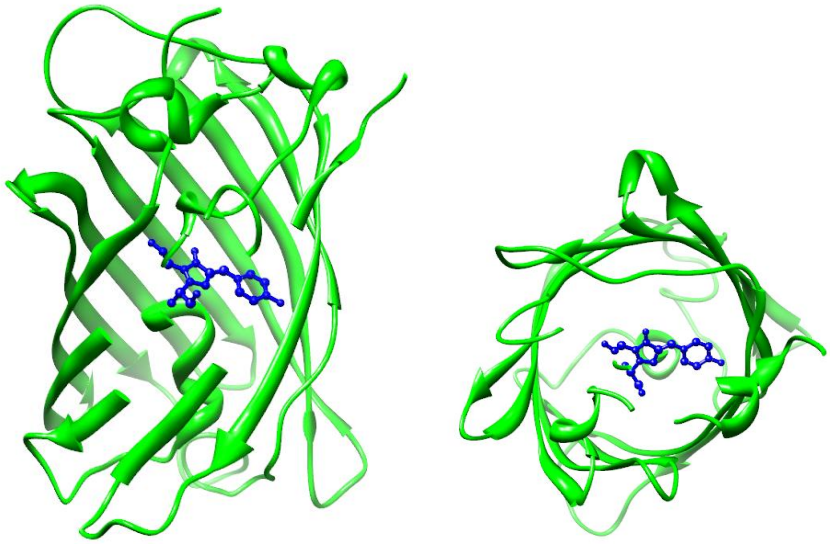

Figure 7. Structure of wtGFP (pdb: 1EMB) (23). The β-barrel structure is shown in green. The fluorophore is shown in blue in ball-and-stick format. Parts of the β-barrel are removed in the image to reveal the structure and position of the fluorophore.

1.5.3. Fluorophore of Green fluorescent protein

Fluorophores of all FPs derived from GFP and coral FPs are planar asymmetric systems sensitive to the excitation light polarization. The best studied is the fluorophore of wtGFP, which is made of three modified amino acids Ser-Tyr-Gly. These amino acids undergo a posttranslational modification and form a 4-(*p*-hydroxybenzylidene)-imidazolidin-5-one fluorophore, which is a planar structure (Figure 8) (22). Absorption of a photon causes an initial alleviation of a conjugated double bond chain in the GFP fluorophore. In aqueous environment, the GFP fluorophore undergoes a rapid cis-trans isomerization, which leads to non-radiative energy dissipation. However, in the GFP molecule, interactions between amino acids forming the β-barrel and the fluorophore prevent cis-trans isomerization of the fluorophore, and create conditions favorable for proton transfer, which brings the GFP molecule into a highly fluorescent excited state (24).

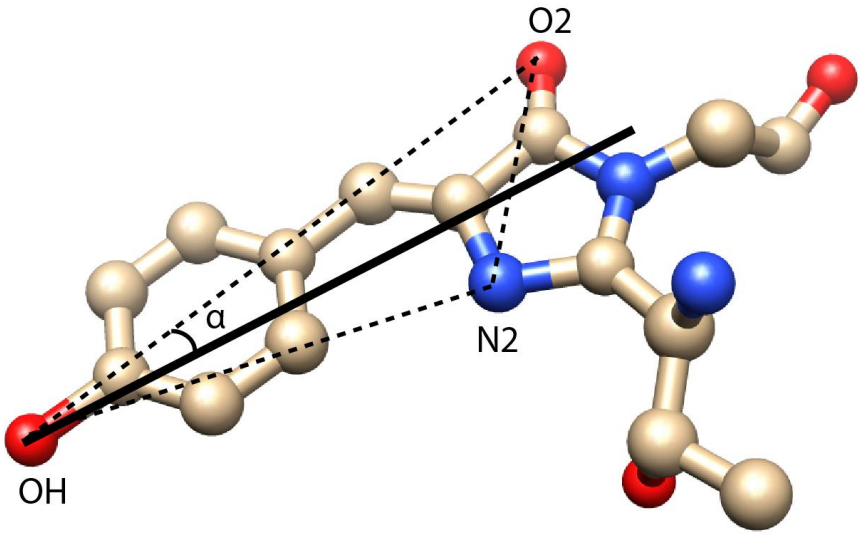

Figure 8. The structure of the wtGFP fluorophore (25). The plane formed by the OH, O2 and N2 atoms is shown by dashed lines. Absorption TDM is shown as a continuous straight line. The angle α is 6.5±5.0^o.

The probability of photon absorption by the GFP molecule depends on the GFP absorption transition dipole moment (TDM) orientation (shown as a continuous line in Figure 8). The TDM is the electric dipole moment associated with transition of a molecule between two (initial and final) states (6). TDM is a vector characterized both by direction and size. The TDM describing single-photon excitation of wtGFP was first characterized by the Boxer group in 2003 (22). The TDM lies in the plane defined by the OH, O2, and N2 atoms of the fluorophore, at an angle α = 6.5±5.0^o from the OH-O2 towards the OH-N2 (25). Probability of light absorption in 1P excitation depends on $\cos^2$ of the angle between the excitation light polarization and the absorption TDM of the FP (26). 2P absorption is described by an absorptivity tensor. In 2P excitation probability of light absorption depends on $\cos^4$ of the angle between light polarization and the absorptivity tensor (6).

Polarization of the emitted fluorescence is defined by the emission TDM which, in wtGFP molecule, is oriented at a 13° angle with respect to the absorption TDM (25). A number of microscopy and spectroscopy techniques take advantage of differences in polarized light absorption by fluorescent molecules or polarization of their fluorescence.

1.6. Microscopy techniques for studies of membrane proteins

There are a number of existing microscopy techniques for studies of membrane proteins in living cells. Each of the techniques described below has its own advantages and limitations, and using a combination of these techniques is often more effective than using a single technique.

Förster Resonance Energy Transfer (FRET). FRET is a method which takes advantage of the effect of non-radiative energy transfer between fluorescent molecules first described by Förster in 1946 (27). A fluorescent molecule which absorbs a photon of excitation light (donor) can transfer energy to another fluorescent molecule of the same or another type (acceptor) in non-radiative manner if the two are sufficiently close to each other and are in favorable orientation (Figure 9). In case of FRET between two different fluorescent moieties, the excitation spectrum of the donor molecule is in the shorter light wavelengths range and of the acceptor in the longer wavelengths range. When the donor molecule is excited by a photon, the energy of this photon is partly transferred to the acceptor molecule and leads to acceptor fluorescence emission. However, efficient FRET is achieved only at short distances (typically ~5 nm for FPs (28)) between fluorophores of the donor and the acceptor (see eq. 1). FRET is widely used for studies of protein-protein interactions, because efficient FRET between protein molecules indicates their close spatial proximity and serves as a strong indication of interaction. However, the method of FRET has its limitations and drawbacks. FRET requires two fluorescent labels in order to determine one protein-protein interaction, which can lead to considerable perturbations of natural systems. Moreover, energy transfer in FRET often has low efficiency, and therefore yields only weak signal intensity. Analysis of FRET experiments can be a tedious procedure requiring multiple correction steps (29). Finally, presence of FRET does not necessarily mean existence of protein-protein interaction - it can also be caused by spatial proximity of proteins not interacting directly.

FRET efficiency (E) is determined by the following equation:

$$E = \frac{1}{1+(r/R_0)^6} \qquad \text{(Eq. 1)}$$

where r is the distance between the donor and the acceptor and R_0 is the Förster distance (the distance at which FRET efficiency is 50%).

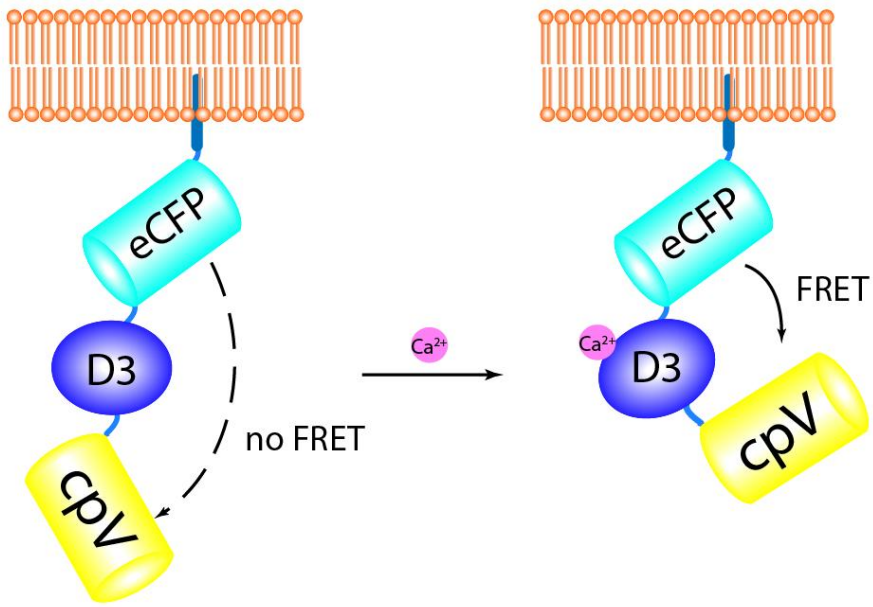

Figure 9. Principle of FRET sensor operation shown on the example of a genetically-encoded Ca^{2+} indicator LynD3cpV (30,31). At low Ca^{2+} concentrations eCFP and circularly-permuted Venus (cpV) moieties are at a considerable distance and efficiency of FRET is very low. High Ca^{2+} concentrations lead to a conformational change in the sensor which brings the two FPs close together and causes an increase in FRET.

Donor recovery after acceptor photobleaching (APB). APB is a method related to FRET, which is often used to verify FRET data. APB is based on bleaching FRET acceptor molecules and measuring accompanying changes in FRET donor fluorescence intensity. If there is efficient FRET occurring between the donor and the acceptor, then bleaching of the acceptor should cause an increase in donor fluorescence, because the donor excitation energy cannot be transferred anymore to the now bleached acceptor. If there is only weak FRET or no FRET, donor fluorescence remains virtually unchanged after acceptor photobleaching. APB often serves as a control for FRET

measurements because it allows distinguishing between specific FRET signal and certain experimental artifacts.

Bioluminescence Resonance energy transfer (BRET). BRET is a method closely related to FRET and using the same principle. BRET utilizes the bioluminescent enzyme luciferase instead of an FP as the donor of photons. Luciferase catalyzes oxidation of luciferin, and one of the products of this reaction is a photon of light. Energy of this photon can be non-radiatively transferred to an acceptor, such as eYFP. BRET has an advantage over FRET because it does not require initial donor illumination and therefore is free of such FRET problems as sample autofluorescence, light scattering, photobleaching and photoisomerization of the donor moiety or photodamage of the cells (32). However, BRET usually provides a very weak signal and similarly to FRET does not directly indicate a physical interaction between molecules.

Fluorescence recovery after photobleaching (FRAP). The FRAP technique is frequently used to determine protein mobility in cells. It can be used both for studies of membrane and cytoplasmic proteins. The method of FRAP is based on bleaching a cellular region and then monitoring fluorescence intensities of this and adjacent regions in order to determine the rate of protein movement in the cell. The FRAP technique can be utilized to determine protein-protein interactions in the plasma membrane of live cells when one of the proteins is immobilized by crosslinking (33), which greatly reduces its mobility. Molecules of the second protein will then diffuse in the membrane at a reduced speed if they interact with the first protein, or remain freely diffusing in the membrane if there is no interaction.

FRAP has several advantages and drawbacks. FRAP allows measuring diffusion rates and thus provides a linkage between fluorescence data and biologically relevant parameters. Suitable control data can be easily acquired for FRAP experiments. However, one has to account for a number of parameters while analyzing FRAP experiments (such as bleaching). Also, sensitivity of the FRAP technique depends on protein diffusion rate and protein-protein interaction kinetics. Heavy perturbation of natural systems is

required when using protein cross-linking and relevance of obtained data to the original system has to be carefully checked and verified.

Total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy takes advantage of the evanescent electric field of light waves for excitation of fluorescent molecules at short distances from a reflecting surface. When light is totally internally reflected at the glass-water interface it produces a standing wave called the evanescent wave which decays exponentially with distance from the interface, and thus only acts at distances of less than ~200 nm (34). The short working distance of the evanescent wave makes TIRF microscopy a valuable tool for studies of membrane proteins located in close proximity to the glass surface.

TIRF microscopy has several useful features crucial for studies of membrane proteins. Due to excitation of molecules only at very short (less than ~200 nm) distances, out-of focus fluorophore bleaching and photodamage are minimized in TIRF microscopy experiments. Exponential decay of the evanescent wave from the surface interface limits the region for fluorescence excitation to the cell membrane, and, therefore, TIRF microscopy allows separating membrane-bound proteins from proteins in the adjacent cytoplasmic region. TIRF microscopy can also be used for visualizing individual molecules in cell membranes.

The main disadvantage of TIRF microscopy is similar to its main advantage, which is imaging at very short distances from the glass surface. One cannot generate an image of the whole cell using TIRF microscopy, and therefore is limited to the thin region of the cell membrane close to the glass surface which does not necessarily represent behavior of the whole cell.

Fluorescence lifetime imaging microscopy (FLIM). The technique of FLIM utilizes fluorescence decay rates of FPs for determination of FP localization and interacting partners. Excited FP molecules can return to the ground state in a variety of ways, one of them being fluorescence emission. Intensity of fluorescence emitted by a FP at a certain time is described by the equation 2:

$$F(t) = F_0 e^{-t/\tau} \quad \text{(Eq. 2)},$$

where t is time and τ is the excited state lifetime (35). Fluorescence lifetime depends on rates of all processes which lead to return of the FP molecules to the ground state. Changes of FP localization in the cell and interactions of the FP molecules with other proteins affect the fluorescence lifetime. Therefore, FLIM is commonly used for studies of protein cellular localization and protein-protein interactions. Importantly, fluorescence lifetime does not depend on fluorescence intensity. This fact makes FLIM the technique of choice for highly scattering samples like the brain tissue (36).

FLIM technique can be efficiently combined with FRET. Such combination helps to overcome some of FRET drawbacks and produce reliable data in samples where acquisition of ratiometric data is a problem.

The main drawback of FLIM is that its results are hard to predict and model because τ is affected by a range of factors, such as FRET, dynamic quenching by small soluble molecules, changes in molecule localization or intermolecular interactions. It is often difficult to interpret the FLIM data, since accurate modeling of multiple processes, which affect the fluorescence lifetime, requires considerable effort and is not always possible.

Despite a number of existing methods for studies of membrane proteins, there is room for improvement of existing techniques, and for development of new methods for studies of membrane proteins in conditions closer to natural than previously possible.

1.7. Polarization microscopy

Probability of photon absorption by the GFP fluorophore depends on the polarization of the excitation light with respect to the GFP molecule. Fluorescence emitted by GFP molecules also has non-random polarization. These features are can be utilized in polarization microscopy techniques. In this section we describe the basic concepts of light polarization and how light polarization can be utilized for studies of membrane proteins in living cells.

1.7.1. Light polarization

Polarization is an intrinsic feature of light waves. Light waves can be represented as plane waves characterized by electric and magnetic field vectors

(Figure 10). These vectors are perpendicular to the direction of light wave propagation and to each other. Light polarization is the direction of the electric field vector orientation of a light wave, which corresponds to the direction of light wave oscillation.

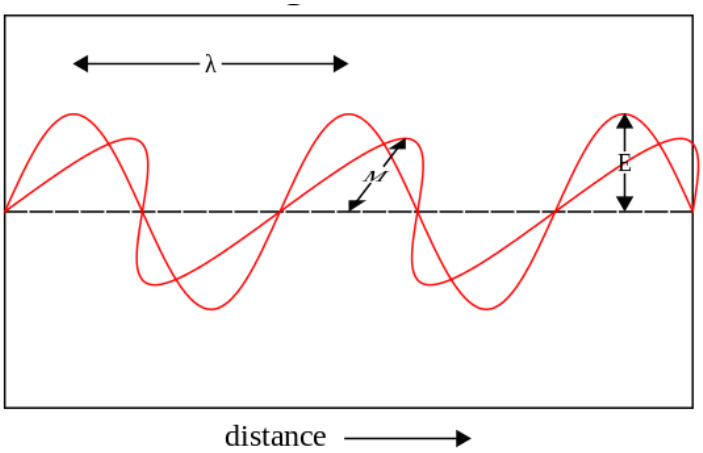

Figure 10. Properties of a light wave. Wavelength (λ) is the spatial period of the wave. Orientation of the magnetic field vector (M) and electric field vector (E) are perpendicular to the direction of light wave propagation and to one another. Polarization of the light wave is the orientation of the vector E. (Artwork courtesy of Gpvos).

Light polarization is commonly visualized by Lissajous figures (6) (Fig.11). In these figures the electric field vector orientation is separated into x and y components and its evolution in time is represented by changes of a point location in space. Projection of obtained 3D trajectories the electric field vector orientation onto 2D space gives rise to an oval shape and its extremes: a line and a circle (Figure 10). In linearly polarized light, orientation of the electric field vector is confined to a single plane along the light wave direction of propagation. In terms of Lissajous figures, the x and y components of the electric field vector are in phase and their projection in 2D space is a line. Vertical and horizontal linear polarizations are commonly distinguished. Circularly polarized light is characterized by constant and uniform change of linear light polarization. In circularly polarized light, the x and y components of the electric field vector have a phase shift of exactly 90°. Circularly polarized light also possesses chirality which indicates the direction of light polarization

rotation. Right-hand and left-hand circular polarizations can be discerned based on the direction of electric field vector rotation. Elliptically polarized light is produced when a non-zero phase shift between the x and y components of the electric field vector is different from 90°. Elliptical light polarization is represented by different kinds of ellipses in 2D projections of Lissajous figures. Non-polarized light consists of light waves with no correlation between electric field vector orientations of different photons. A random combination of polarizations of light waves gives rise to truly non-polarized light. Partial correlation between polarizations of photons produces partially polarized light. Technology applications of light polarization range from studies of surface imperfections to 3D cinema. A number of microscopy techniques also take advantage of light polarization.

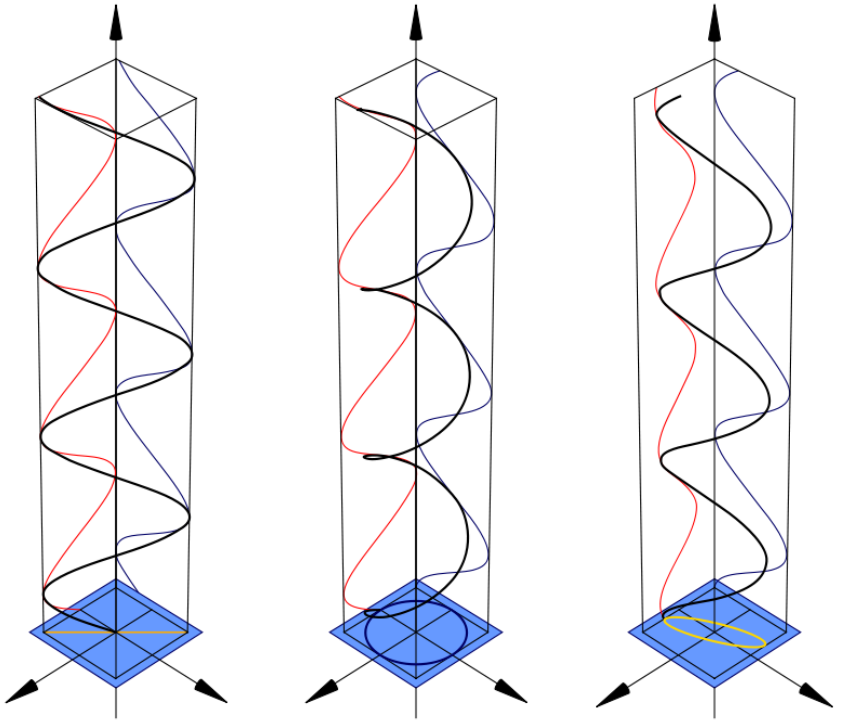

Fig. 11. Light polarization represented as Lissajous figures. A) Linearly polarized light. B) Circularly polarized light. C) Elliptically polarized light. (Artwork courtesy of Inductiveload)

1.7.2. Polarization microscopy techniques

Polarization microscopy includes a range of optical imaging techniques which utilize polarization of excitation light or emitted fluorescence for

obtaining information about the studied object. Two main groups of polarization microscopy techniques are:

1) fluorescence polarization microscopy
2) linear dichroism microscopy

The first group includes techniques which use the polarization of emitted fluorescence for obtaining data about conformation, orientation, rotational rate, and interactions of the studied object. The second group includes techniques which use the differences in absorption of polarized light of different linear polarizations for obtaining information on the orientation and conformation of the studied molecules.

1.7.2.1. Fluorescence polarization microscopy

Fluorescence polarization microscopy takes advantage of unequal intensities of fluorescence emitted by a sample in different polarization axes (fluorescence anisotropy) (37). Fluorescence anisotropy is present when fluorescence emitted by a fluorophore upon excitation with linearly polarized light is preferentially horizontally or vertically polarized. Fluorescence anisotropy is commonly used for determination of rotation rate of molecules and their orientation. In the most common fluorescence polarization microscopy setup, linearly polarized light is used for fluorescence excitation, and emitted fluorescence is separated into its horizontal and vertical polarization components (by a polarizing beamsplitter and polarizers), whose intensities are then detected. The extent of fluorescence polarization is commonly quantitated in terms of fluorescence anisotropy (r), which is described by the following equation 3:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad \text{(Eq. 3)},$$

where $I_{\parallel}$ denotes intensity of fluorescence polarized parallel to the excitation light polarization, and $I_{\perp}$ signifies intensity of fluorescence polarized perpendicularly to the excitation light polarization (38).

Fluorescence anisotropy measurements can be used for studies of kinetics of enzymatic reactions, membrane or cytoplasm viscosity, or protein-protein interactions (6). Fluorescence anisotropy can yield information about interactions of small molecules with enzymes, especially if a fluorophore is

attached to the small molecule, because its rotation rate is strongly affected by interactions with the enzyme. Changes of viscosity of cytoplasm or membranes of live cells can be detected by measirung rate of rotation of fluorescent molecules localized in these cellular compartments (39). Fluorescence anisotropy measurements can be used both for *in vitro* and *in vivo* studies.

Fluorescence anisotropy of wtGFP was first observed by the Jovin group in 2000 (40). Since then fluorescence anisotropy of FPs has been used for studies of membrane microenvironment and protein-protein interactions in live cells (39,41). A combination of FRET and FP fluorescence anisotropy measurements is often utilized for studies of protein-protein interactions (42,43) because FP fluorescence anisotropy can be used for reliable detection of both homoFRET and heteroFRET (43). Therefore, FP fluorescence anisotropy measurements have multiple applications for live cell studies.

1.7.2.2. Linear dichroism microscopy

Linear dichroism (LD) is a property of chemical compounds to preferentially absorb linearly polarized light of certain polarization. LD is the difference in absorption of light polarized parallel and perpendicular to a reference direction (26) (Eq. 4)

$$LD = A_{\parallel} - A_{\perp} \qquad \text{(Eq. 4)},$$

where $A_{\parallel}$ and $A_{\perp}$ are absorptions of light oriented parallel and perpendicular to the reference direction, respectively. In fluorescent molecules, LD can be conveniently measured through detection of fluorescence intensity (fluorescence detected linear dichroism). The extent of LD can be expressed in terms of a dichroic ratio (r), obtained by calculating the ratio of fluorescence intensities measured using excitation with horizontally polarized light (F_{h}) and vertically polarized light (F_{v}) (Eq. 5) (6)

$$r = \frac{F_h}{F_v} \qquad \text{(Eq. 5)}.$$

Although the dichroic ratio and fluorescence anisotropy are often denoted by the same letter "*r*", they are distinct qualities and not to be confused. Unlike fluorescence anisotropy, LD reports on absorption of linearly polarized light by

fluorophore molecules rather than polarization of the fluorescence emitted by them.

LD is present in molecules which maintain well-defined spatial orientation. LD is not observed in molecules in solution or cytoplasm due to presence of molecules in various orientations and inability to resolve them individually because of the diffraction limit. However, this feature makes LD a valuable tool for studies of molecules which have a well-defined orientation, such as membrane proteins. LD is present not only in membrane-bound molecules, but in any molecules spatially aligned using Couette flow (44) or embedded in a stretched thin film (26).

LD measurements can be used as a spectroscopic technique for determination of molecular structure and interactions of the studied molecule (e.g. DNA) with ligands or enzymes *in vitro* (45).

Live cell LD microscopy usually relies on fluorescence-detected LD (46), where LD of the studied molecule is determined by measuring its fluorescence output when using horizontal and vertical excitation light polarization. LD microscopy using fluorescent dyes has been applied to studies of lipid membrane organization in mammalian cells (47) and determination of plant cell wall ultrastructure (48).

Measurements of FP LD have not been widely used for studies of live cells. LD in wtGFP was first observed by Inoue and Shimomura (49) who used this property together with fluorescence anisotropy for determination (incorrect, due to wrong assumptions on structure of wtGFP crystals) of orientation of wtGFP molecules in a crystal. Although FP fluorescence anisotropy has became routinely used for studies of live cells (50), FP LD microscopy has not received wide popularity, and its applications to live cell studies have been limited to determination of septin orientation in yeast (51). We decided to take advantage of FP LD measurements combined with 2P microscopy to obtain structural and functional information about membrane proteins in live cells.

We have decided to use LD measurements for a number of reasons. LD measurements yield information about protein conformation and orientation and do not require maintaining polarization of the emitted fluorescence along its beampath to the detector. In most microscopes collection of the emitted fluorescence occurs through a high NA objective lens which scrambles

polarization of the collected light. Therefore, building a sensitive microscopy setup for fluorescence anisotropy detection is a challenging task. Since LD measurements do not depend on polarization of emitted fluorescence, they provide more accurate information when using high NA objective lenses, and are technically easier to implement (at least in their basic form) on regular microscopes.

2P excitation increases sensitivity of LD measurements. Pulsed IR laser light utilized in 2P microscopy is highly linearly polarized. Therefore, there is no need for additional equipment for obtaining linear light polarization. Limitation of 2P excitation to a small focal volume minimizes the number of molecules excited by light of undesired polarizations. Finally, 2P excitation yields the highest selectivity of FP excitation due to necessity of two absorption events for excitation of a single molecule of a FP. All these reasons make a combination of 2P microscopy and LD microscopy a promising approach for creating a tool for studies of membrane protein structure and function.

1.8 G protein signaling cascade

In our studies of membrane proteins we focused on the G protein signal transduction system. This system is of high scientific, medical and pharmacological importance. Two Nobel prizes have already been awarded for elucidation of the G protein and GPCR structure and function (Rodbell and Gilman 1994, Lefkowitz and Kobilka 2012). About 30-50% of all modern drugs target members of the G protein signaling cascade, particularly G protein-coupled receptors (GPCRs) (52).

Despite much research into G protein signaling, its molecular mechanism is not yet fully understood. We have utilized the technique of 2PPM to address some of the most controversial questions of interactions between members of the G protein signaling cascade.

1.9. Members of G protein signaling cascade

The G protein signaling system is a key player of cellular signal transduction. It serves for transduction and amplification of signals from a multitude of physical and chemical extracellular stimuli including hormones, neurotransmitters, light, odorants, and others.(53) The G protein signal transduction system is critical for cellular communication with extracellular environment and adaptation to its changing conditions. G protein signal transduction systems are present in animals, plants and yeast (54). In humans, G proteins regulate numerous processes including functional activity of the heart, kidneys, sensory organs, gastrointestinal and nervous systems (55). Members of the G protein signaling cascade are important drug targets. However, the precise mechanism of the G protein cascade signaling remains unclear (56).

The G protein signal transduction cascade consists of three main components: a G protein-coupled receptor (GPCR), a G protein, and an effector. Extracellular ligands, such as hormones, bind and activate transmembrane molecules of GPCRs which undergo conformational changes and, in turn, bind and activate G proteins located on the inner side of cellular plasma membrane. G protein activation is accompanied by a release of a guanosine diphosphate (GDP) molecule bound to the Gα subunit of G proteins in the inactive state and binding of a guanosine triphosphate (GTP) molecule instead. Activation of multiple G protein molecules by a single molecule of a

GPCR leads to signal amplification. Activated G proteins transduce the signal to downstream effectors, which can be intracellular enzymes producing second messengers, or ion channels. Production of second messenger molecules such as 3'-5'-cyclic adenosine monophosphate (cAMP), diacyl glycerol (DAG), or inositol triphosphate (IP_3) leads to complex modification of intracellular metabolism by virtue of modulation of activity of multiple enzymes. Transduction along the G protein cascade leads to signal de-specification because information from a multitude of extracellular stimuli is transduced by only few types of second messengers. A schematic of the G protein signaling cascade is shown in Figure 12.

Deactivation of G proteins occurs primarily due to intrinsic GTPase activity of Gα subunits. Hydrolysis of the G protein bound GTP molecule into GDP and a phosphate ion returns a G protein to its inactive state. The GTPase activity of G proteins is regulated by a number of regulatory proteins.

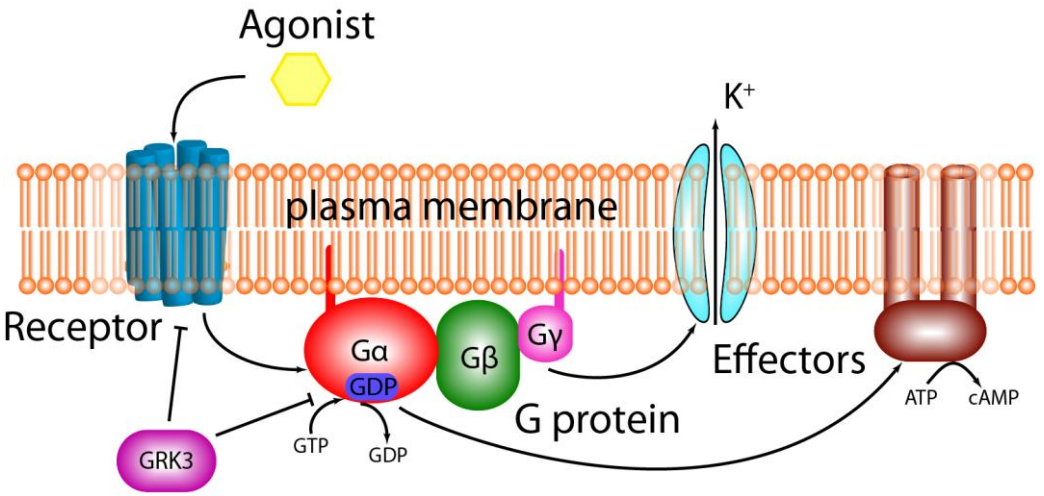

Figure 12. G protein signaling cascade. The members of the cascade include extracellular agonists, transmembrane GPCRs, heterotrimeric G proteins, effectors, which produce second messengers, and regulators of signaling (e.g. GRK3).

1.9.1. GPCRs

GPCRs represent a diverse family of molecules, encoded in humans by more than 800 genes, united by common structural features and signaling mechanisms (55). GPCRs are transmembrane proteins located in the cellular plasma membrane. They are usually divided into five main classes (57):

1) rhodopsin-like receptors (includes 85% of all GPCRs)
2) secretin-like receptors
3) metabotropic glutamate/pheromone receptors
4) adhesion receptors
5) frizzled/taste-2 receptors.

These classes of GPCRs evolved independently and do not possess substantial sequence homology. However, they all share a common 7 transmembrane domain structure which allows them to efficiently interact with G protein α subunits. The structure of a representative GPCR (β2 adrenergic receptor) in shown in Figure 13 (58,59). The N-terminal segment of the GPCR structure is located outside the cell, while the C-terminus is in the cytoplasm. Seven α-helices penetrate the membrane and three interhelical loops are located on each side of the plasma membrane.

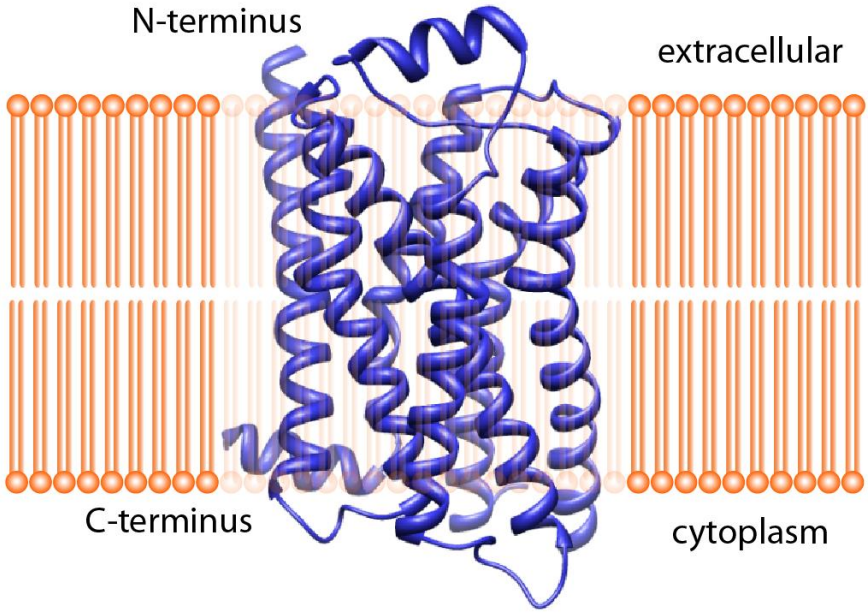

Figure 13. The structure of β2 adrenergic receptor (pdb: 2RH1) (58). The receptor consists of seven transmembrane domains and six interdomain loops (three loops on each side of the membrane). The N-terminal part of the receptor faces extracellular environment. The C-terminus faces cytoplasm. Binding of extracellular ligands leads to conformational changes, which promote binding and activation of G proteins.

GPCR of all five classes share a common mechanism of function (60). The extracellular part of the GPCR molecule, including its N-terminus, participates in ligand recognition and binding. Conformational changes induced by the ligand binding are transferred to the intracellular part of the GPCR via transmembrane α-helices. The intracellular loops and C-terminus of the GPCR then adopt a conformation favorable for G protein activation, complementary to the C-terminus of a Gα subunit (61). Binding of the G protein molecule to the activated GPCR leads to release of GDP from the Gα subunit and triggers the downstream signaling.

The individual GPCR classes possess class-specific features (62). Rhodopsin-like receptors have a short N-terminus and contain DRY and NSXXNPXXY motifs in their structure, which are required for interaction with G proteins and receptor ground state stabilization (63). Unlike rhodopsin-like receptors, secretin receptors have a long N-terminus, which allows them to interact with peptide hormones. Members of the metabotropic glutamate receptor class possess a "Venus flytrap" ligand binding domain, which closes around the ligand. Adhesion receptors have specific extracellular determinants for cell-cell binding. Frizzled receptors have a Cys-rich N-terminal domain suited for binding glycoproteins and play a role in sonic hedgehog pathway (62).

In our research we mainly focused on two receptors: α2A-adrenergic receptor (α2A-AR), which is a rhodopsin-like receptor, and γ-aminobutyric acid receptor B ($GABA_B$), which belongs to metabotropic glutamate receptor class (57). These receptors differ in their structure and in their mechanism of interaction with ligands. Unlike α2A-AR, $GABA_B$ receptor is an obligatory heterodimer composed of two subunits ($GABA_B1$ and $GABA_B2$) that together form a functional receptor. Although both α2A-AR and $GABA_B$ signal through similar G proteins, due to the dissimilar receptor structures, functionally important differences in their interactions with G proteins can be expected.

1.9.2. G proteins

G proteins are guanine nucleotide-binding proteins. There are two main types of G proteins: monomeric and heterotrimeric. Monomeric G proteins (also referred to as small GTPases) are structurally similar to α subunits of

heterotrimeric G proteins and similarly to Gα subunits possess a GTPase activity. Heterotrimeric G proteins consist of α, β and γ subunits. In humans, there are 21 subtypes of Gα subunits encoded by 16 genes, 6 subtypes of Gβ subunits encoded by 5 genes, and 12 subtypes of Gγ subunits (64). In the resting state, Gα subunits are associated with Gβγ dimers through interaction with Gβ subunits (65). The interaction between Gβ and Gγ subunits is very stable, and Gβγ dimers do not dissociate in natural conditions (66). Gα subunits generally lack specificity in binding particular types of Gβ subunits or Gβγ dimers (with the exception of Gβ5) (67). Both Gα subunits and Gβγ dimers regulate activity of intracellular effectors. The structure of a representative G protein heterotrimer Gαi1β1γ2 (PDB: 1GP2) (68) is shown in the Figure 14.

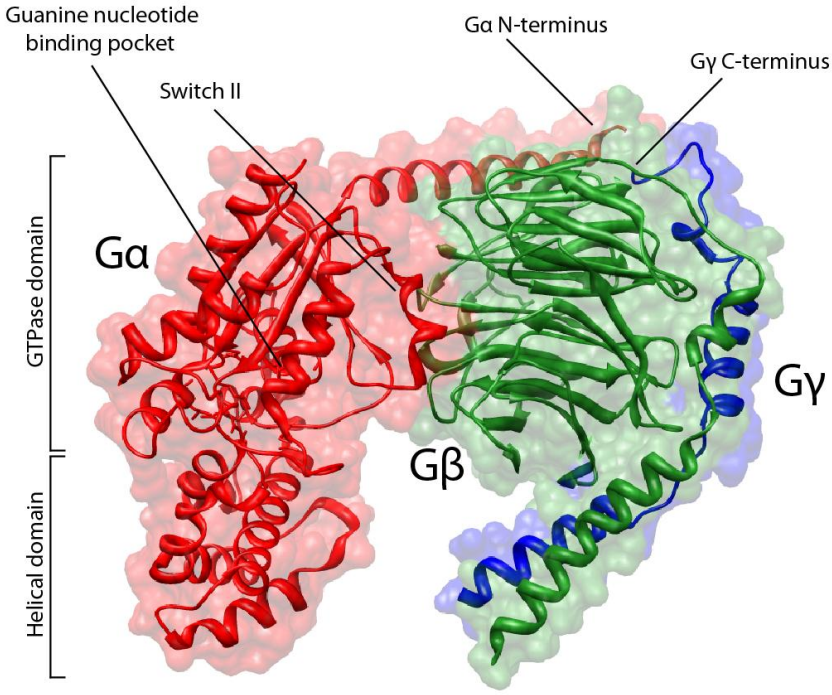

Figure 14. Structure of a G protein heterotrimer (pdb: 1GP2) (68). The heterotrimer consists of Gαi1, Gβ1 and Gγ2 subunits. The Gαi1 subunit directly interacts with the Gβ1 subunit but not with Gγ2. The guanine nucleotide binding pocket and Switch regions of Gα play a crucial role in G protein activation. Membrane-binding lipid tags are located at the N-terminus of the Gαi1 subunit and the C-terminus of the Gγ2 subunit.

1.9.2.1. Gα subunits

Gα subunits of G proteins bind guanine nucleotides and determine specificity of G protein interactions with GPCRs. Four families of Gα subunits can be discerned, as summarized in Table 1 (69). Gα subunits consist of two domains: the GTPase domain and the helical domain. The GTPase domain contains the guanine nucleotide binding site and a Mg^{2+} binding site. This domain can bind both GDP and GTP, and possesses the GTPase enzymatic activity (65). Mg^{2+} is an essential cofactor for the GTPase activity of the Gα subunit. Since Gα subunits possess a GTPase activity, they self-inactivate by hydrolyzing GTP, which regulates the duration of G protein activation. The helical domain takes part in stabilizing the guanine nucleotide binding, serving as a "lid" covering the GTPase nucleotide binding pocket of the GTPase domain (62). The helical domain also takes part in GDP release and likely determines specificity of G protein interactions with effectors (70). The N- and C-termini of the Gα subunit play important roles in the subunit's cellular localization and functional activity (71,72). The N-terminus of Gα subunits undergoes posttranslational lipidation and promotes localization of the protein to the inner leaflet of the cellular plasma membrane. All Gα subunits (except Gαt) are palmitoylated at one or several sites (71). Members of the Gαi/o family are also myristoylated, which irreversibly localizes them to the membrane (73). Both N- and C-termini of Gα subunits interact with GPCRs and play role in activation of G proteins. It has been shown that a C-terminal part of Gα subunits is responsible for GPCR recognition specificity (72). Replacement of a few C-terminal amino acids may drastically affect the selectivity of G protein interactions with particular GPCR types.

Gα subunits interact with Gβγ dimers through the Gβ subunit. No direct evidence of Gα-Gγ interactions has been found yet (67). Two major sites of Gα-Gβ interactions on the Gα subunit are located at the GTPase domain and the N-terminus (68). The GTPase domain contains three flexible loops near the guanine nucleotide binding site (Switches I, II and III). Switches I and II of Gα bind to the Gβ subunit; their considerable displacement upon G protein activation leads to disruption of the interaction with the Gβ subunit. The N-terminal α-helix of Gα subunits is located between the cell membrane and the Gβ subunit. This α-helix either pulls the Gβγ dimer away from the Gα GTPase

domain upon GPCR-mediated activation (lever-arm theory), or pushes on the Gβ subunit (gear-shift theory) (62), which leads to GDP release from the Gα subunit. Disruption of the Gα-Gβ interaction in the Switch regions of Gα leads to formation of an interface for an interaction with effectors on the Gα and Gβ subunits. The most common intracellular effectors regulated by G proteins are presented in Table 1.

Table 1. Families of Gα subunits (74).

Family	Members	Effectors	Activity
Gαi/o (inhibitory)	Gαt1, Gαt2, Gαi1, Gαi2, Gαi3, Gαo, Gαz, Gαgust	Adenylyl cyclase cGMP Phosphodiesterase GIRK (Gβγ effector)	↓ ↑ ↑
Gαs (stimulatory)	Gαs, Gαolf	Adenylyl cyclase	↑
Gαq/11	Gαq, Gα11, Gα15/16	PLC	↑
Gα12/13	Gα12, Gα13	RhoGEF	↑

In our research we focused on four members of the inhibitory Gαi/o family of heterotrimeric G proteins, namely, Gαi1, Gαi2, Gαi3 and Gαo. These Gα subunits inhibit adenylate cyclase (AC) activity and reduce intracellular cAMP concentration. Gβγ dimers derived from activated G protein heterotrimers containing Gαi/o subunits stimulate GIRK channel activity. An important feature of Gαi/o subunits is their irreversible inactivation by Pertussis toxin (PTX). PTX catalyzes Gαi/o subunit ATP-ribosylation and prevents Gαi/o interaction with GPCRs (75). A single point mutation of a C-terminal cysteine of Gαi/o subunits allows making these proteins insensitive to PTX (76). Gαi/o mutants insensitive to PTX are often used in live cells experiments, because application of PTX allows studies of exogenously expressed (PTX-resistant) Gi/o proteins without interference from endogenous (PTX-sensitive) Gi/o proteins (77).

1.9.2.2. Gβγ dimers

Gβ subunits form stable complexes with Gγ subunits. The Gβγ dimers dissociate only in denaturating conditions (67). All Gβ subunits adopt a seven-

bladed β-propeller structure (made of WD40 repeats), with an N-terminal α-helix (78). The β-propeller surface of Gβ subunits interacts with Gα subunits and multiple effector and regulatory proteins. Gβ subunits do not possess any membrane-targeting motifs (71). Gβ subunits can interact with different Gα subunits and generally do not show preference for interactions with a particular type of Gα subunits (79).

Gγ subunits are small proteins (7-8 kDa) which form two α-helices connected by a loop. The N-terminal α-helix of the Gγ subunit forms a coiled-coil structure with the N-terminus of Gβ, and the C-terminal α-helix of Gγ interacts with the β-propeller of Gβ (68). All Gγ subunits are posttranslationally isoprenylated at their C-terminus. Either farnesyl (15-carbon) of geranylgeranyl (20-carbon, all other nine Gγ types) lipid tag is attached to the C-terminal CaaX box of Gγ subunits (71). The type of the isoprenoid tag specifically corresponds to the type of Gγ subunit: Gγ1, Gγ9 and Gγ11 are farnesylated, and all other nine types of Gγ subunits are geranylgeranylated (74). Gγ subunits provide a membrane-binding anchor for Gβγ dimers. Evidence for interactions with Gα subunits being required for plasma membrane attachment of Gβγ dimers is contradictory (73,80).

Gβγ dimers regulate activity of multiple effectors. Initially, no functional activity was attributed to Gβγ dimers, and their role in signaling was limited to passive dissociation from activated Gα subunits and re-association with them for G protein inactivation (75). However, Gβγ dimers were later shown to directly regulate activity of multiple effectors, such as GIRK channels and PLCβ (67). Specificity of Gβγ interactions with effectors is defined by the type of Gβ and Gγ subunits, and the type of Gγ isoprenoid tag (79). Interactions of Gβγ dimers with effectors are regulated by cell-specific (or tissue-specific) expression of Gβ and Gγ subunit types, precoupling of Gβγ dimers to effectors and possibly by dissociation or rearrangement of G protein heterotrimers upon activation (81).

1.9.3. Regulators of G protein signaling

G protein signal transduction is affected by multiple regulatory proteins, such as GTPase-activating proteins (GAP), regulators of G protein signaling (RGS), activators of G protein signaling (AGS) or GPCR-kinase (GRK)

proteins. These regulators can either promote activation (AGS) (82) or inactivation (GAP, RGS, GRK) of G proteins (62). In our research we utilized a peptide derived from C-terminal fragment of GRK3 (GRK3ct) (83) in order to determine whether G protein heterotrimers physically dissociate in live cells.

GRKs are intracellular regulators of G protein signaling that bind GPCRs, as well as activated G protein subunits. GRKs phosphorylate GPCRs and prepare them for β-arrestin binding (84). Thus, GRK binding leads to receptor and G protein inactivation. The first structure of GRK (GRK2) was determined by the group of J.J. Tesmer in 2003 (85,86). This structure showed that GRK2 contains three domains, which have binding sites for different proteins and perform distinct functions. One domain binds molecules of GPCRs, another binds $G\alpha_{GTP}$ subunits, and a third one binds Gβγ dimers. Binding of all these molecules leads to inactivation of both GPCRs and G proteins. Importantly, sites of GRK2 interaction with the Gβγ dimer largely overlap with sites of Gα subunit interaction with Gβγ (87). The overlap includes both interaction interfaces of Gα subunit with Gβγ dimer (Switch region II and N-terminus). Therefore, a Gβγ dimer can be bound to either a Gα or to GRK2, but not both at the same time. Probes based on C-terminal Pleckstrin Homology domain of GRK3, which is highly homologous to GRK2, are useful indicators of G protein heterotrimer dissociation upon activation (83).

1.9.4. G Protein Effectors

G protein effectors are molecules whose activity is regulated by G proteins. Effectors can be enzymes producing second messengers, or ion channels. The most common effectors include adenylyl cyclase (AC), phospholipase Cβ, cGMP phosphodiesterase, Ca^{2+} channels, G protein-regulated inward rectifying K^+ (GIRK) channels, and p115RhoGEF.

The first effector discovered was the AC (75). Consequently G proteins are often divided into Gs (stimulatory) and Gi (inhibitory) families, based on their effect on the AC (88). AC catalyzes production of cAMP from ATP. cAMP is a second messenger. A cAMP concentration increase leads to stimulation of activity of protein kinase A (PKA) and EPAC proteins, and to opening of ion channels (89).

The Gq/11 family of G proteins stimulate activity of phospholipase Cβ (PLCβ), which catalyzes breakdown of phosphatidylinositol bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol triphosphate (IP_3). DAG stimulates protein kinase C (PKC), and IP3 causes release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm.

Activation of RhoGEF by the G12/13 protein family leads to stimulation of small cytoplasmic GTPase Rho. Rho, in turn, modulates the activity of cytoskeleton-regulating proteins. The activity of AC, PLCβ and RhoGEF is regulated directly and selectively by Gα subunits (78).

Other effectors are regulated by Gβγ dimers. Among these, the most studied are GIRK channels. These channels are present in the heart and the nervous system. When activated by Gβγ dimers, they become permeable to K^+ ions, leading to hyperpolarization of the cell (90). Interestingly, GIRK channels can be activated only by Gβγ dimers arising from activated Gi/o proteins, but not from G proteins of other families (67). Since Gα subunits of different families do not exhibit pronounced selectivity in binding particular Gβ or Gγ subtypes, it is unclear what determines the specificity of Gβγ-GIRK interactions. It has been proposed that Gi/o protein heterotrimers form stable complexes with GIRK channels in the inactive state, with spatial proximity then being the key determinant for the Gβγ-GIRK interaction selectivity (91). Alternative or additional mechanisms for this selectivity have also been proposed, including differences in dissociation or re-arrangement of G protein heterotrimers of different families upon activation (92,93).

1.10. Previous studies of G protein signaling

Despite numerous studies of the G protein signaling cascade, its molecular mechanism of function is not entirely clear. We have addressed three important controversial issues:

1. Do Gi/o protein heterotrimers dissociate upon activation
2. What is the functional significance of Gi/o protein dissociation?
3. Do Gi/o proteins interact (precouple) with their cognate GPCRs in the inactive state?

1.10.1. Gi/o protein activation

Stimulation of a GPCR molecule by a chemical ligand or a physical stimulus leads to changes in receptor conformation which allow the GPCR to bind and activate molecules of G proteins. Activation of a G protein molecule leads to the release of a GDP molecule bound to the Gα subunit, and capture of a GTP molecule from the cytoplasm. This nucleotide exchange promotes further changes in the G protein heterotrimer. It was thought originally (75) that after the nucleotide exchange, G protein heterotrimers dissociate into free $G\alpha_{GTP}$ subunits and Gβγ dimers. However, this postulate has been questioned in a number of studies (33,92,94,95) which suggested a rearrangement, rather than dissociation, of G protein heterotrimers upon activation.

The long-standing question whether G protein heterotrimers dissociate upon activation remains widely disputed. *In vitro* experiments largely support G protein dissociation upon activation. It has been shown with purified G proteins that binding of non-dissociating GTP analogs (such as GTPγS) and Gβγ dimers to Gα subunits exhibits negative cooperativity, which indicates dissociated state of activated Gα subunits and Gβγ dimers (75). A. Gilman and co-workers have shown that individual G protein subunits are able to modulate effector activity *in vitro*, concluding that heterotrimer formation was not required for regulation of effector activity (96). Crystal structures of G proteins show that conformation changes in Switch regions of the activated GTPγS-bound Gα subunit eliminate the Gβ-binding surface (62). All these data suggests dissociation as the universal feature of G protein heterotrimer transformation upon activation.

However, some phenomena cannot be fully explained by this theory. In particular, it remains unclear why only Gβγ dimers derived from activated Gi/o proteins, but not from other types of G proteins, are able to stimulate GIRK channel activity (97). It should be possible to address this and other issues by experiments using live cells.

Unfortunately, experiments on Gi/o proteins in live cells have yielded inconsistent results and raised further concerns over *in vitro* studies. Studies carried out by the Bünemann group showed an increase in FRET between the FP-labeled Gαi (FP inserted into the αa-αb loop, after Leu 91) and Gβγ subunits upon G protein activation, suggesting activated heterotrimer rearrangement

(92,98). Similar results were obtained by M. Bouvier and co-workers using the BRET technique on Gαi subunits labeled at a different site (Gly60) (95). However, a study by Gibson and Gilman using G protein subunits tagged with an FP at Ala121 in the αb-αc loop showed a decrease in FRET, consistent with heterotrimer dissociation (99). It appears that results of FRET and BRET experiments depend on the label insertion site. A different approach to the problem could be more fruitful. Such a new approach was used by the group of N. Lambert who used the FRAP technique to show that Gi/o proteins likely dissociate upon activation, while the Gs protein heterotrimer undergoes rearrangement (33,93). These results strongly contradicted *in vitro* data, as the Gαs protein had been shown *in vitro* to exhibit a lower affinity to Gβγ dimers than Gαi/o proteins (75). Also, BRET results obtained by the same group using membrane-bound GRK3ct probe were consistent with dissociation of heterotrimers containing different FP-tagged Gαi1 constructs, including those showing an increase in FRET (or BRET) between G protein subunits upon activation (83). Since different techniques have yielded mutually contradictory data, the question whether Gi/o proteins undergo rearrangement or dissociation upon activation has remained open and requires more thorough studies.

An important drawback of published approaches to the question of G protein dissociation/rearrangement upon activation has been the need for multiple fluorescent labels or other modifications to the system. An important strength of the 2PPM technique is its need for only a single fluorescent moiety in order to report on a protein-protein interaction. Thus, using 2PPM, only a single FP label is needed for detection of interactions between Gα and Gβγ subunits, allowing studies of G protein activation in conditions closer to natural than previously possible.

1.10.2. Studies of interaction between GPCRs and Gi/o proteins in inactive state

Interactions between G proteins and GPCRs play an important role in the dynamics and specificity of signal transduction. Two major models have been proposed concerning the GPCR-G protein interactions: the collision coupling model and the precoupling model.

The collision coupling model postulates that GPCRs and G proteins freely diffuse in the cell membrane and interact (couple by collision) only upon activation of GPCRs (100,101). This model is supported by a number of studies in live cells (102,103). However, this model does not fully explain fast rates (tens of milliseconds (103)) of specific interaction between activated GPCRs and G proteins (104).

An alternative precoupling model suggests that GPCRs form stable complexes (precouple) with G proteins, thereby securing specificity of GPCR-G protein interaction and ensuring its fast rate (62). This model is supported by a number of in live cells (95,105,106) and *in vitro* (107). The precoupling model, however, does not sufficiently explain signal amplification upon signal transduction from GPCRs to G proteins (62).

Since both original models fell short in explaining some of the observed G protein signaling features, other theories emerged suggesting that in the inactive state GPCRs and G proteins might be spatially confined to cell membrane compartments (e.g. lipid rafts or caveolae) (108) or they may switch from precoupling to collision coupling upon GPCR activation (104). In general, an ideal theory should simultaneously account for specificity and fast kinetics of GPCR-G protein interaction, sharing of pools of G proteins and effectors, and signal amplification along the G protein signaling cascade (104). The mode of GPCR interaction with G proteins may also depend on the cell type and organism as well as a particular type of a G protein or a GPCR; therefore, interactions between these molecules do not necessarily obey one universal model.

We have studied interaction of two GPCRs α2A-AR and $GABA_B$ with Gi/o proteins. Coupling between α2A-AR and Gi/o proteins is a highly disputed and controversial issue. FRET studies by the Tinker group in live cells suggested formation of a stable complex between the Gαi1β1γ2 protein heterotrimer and α2A-AR (105). Similar results were obtained by the Bouvier group using BRET technique (95). However, the same experimental model with differently tagged constructs used by the Bünemann group showed no detectable precoupling between the Gαi1β1γ2 heterotrimer and α2A-AR (103). FRAP studies by the Lambert group also suggested no detectable precoupling for these molecules, although could not completely exclude it (102). Incoherent

data obtained in live cells studies may be attributed to modification of signaling molecular mechanisms by labeling or cross-linking of GPCR or G protein molecules, or protein overexpression. We decided to study the interaction of α2A-AR with Gi/o proteins to resolve this long-disputed issue. We also aimed to determine the interaction mode between G proteins and $GABA_B$ receptor, whose mode of coupling to G proteins is much less studied (109), although a FRET study suggested possible precoupling of $GABA_B$ to Gi/o proteins (110). Utilization of α2A-AR and $GABA_B$ should allow us to determine if Gi/o proteins have similar coupling properties for GPCRs of different classes. In our studies we used the 2PPM technique which allows direct detection of GPCR-G protein interaction using a single FP label. We studied the GPCR-G protein coupling process in less perturbed system than in previous studies.

2. Materials and methods

2.1. Molecular biology

The constructs used in our studies were kind gifts or were purchased from Addgene (USA) or the Missouri S&T cDNA Resource Center (USA). All mutants of the constructs were created as follows. PCR primers bearing desired mutations were designed in Genetool and ApE and synthesized by Sigma (Germany). PCR reactions were performed using Phusion Hot Start DNA Polymerase (NEB, USA) according to the manufacturer's instructions. PCR products were purified on an agarose gel and extracted using the QiaQuick gel extraction kit (Qiagen, USA). Prepared linear DNA was 5' phosphorylated with T4 polynucleotide kinase (NEB, USA) and circularized with T4 DNA ligase (NEB, USA). The constructs were transformed into *E. coli* XL-1 Blue competent cells (Life Technologies, USA), which were grown on agar plates supplemented with an appropriate antibiotic, and individual colonies were analyzed. The colonies were inoculated into liquid Luria-Bertani medium with the antibiotic and incubated overnight at 37 ^{o}C. Plasmid DNA was prepared from bacteria using the QiaPrep Spin miniprep kit (Qiagen, USA) according to manufacturer's protocol. Correctness of all mutants was verified by sequencing (LGC Genomics, Germany).

2.2. Mammalian cell culture and transient transfection

HEK293 cells were cultivated in 25 cm^2 flasks in Dulbecco modified Eagle Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum and antibiotics in the atmosphere of 5% CO_2 at 37 ^{o}C. We transiently transfected the cells with Lipofectamine 2000 (Life Technologies, USA) according to the manufacturer's protocol. All constructs were transfected in equimolar quantities, except for the GRK3ct-FP constructs, which were transfected at 0.8 equivalents. For microscopy experiments, DMEM was replaced with HEPES-buffered Hanks balanced salt solution with 1% Glucose (HHBSS, Life Technologies, USA). Microscopy experiments were performed 48 hours after transfection, at room temperature.

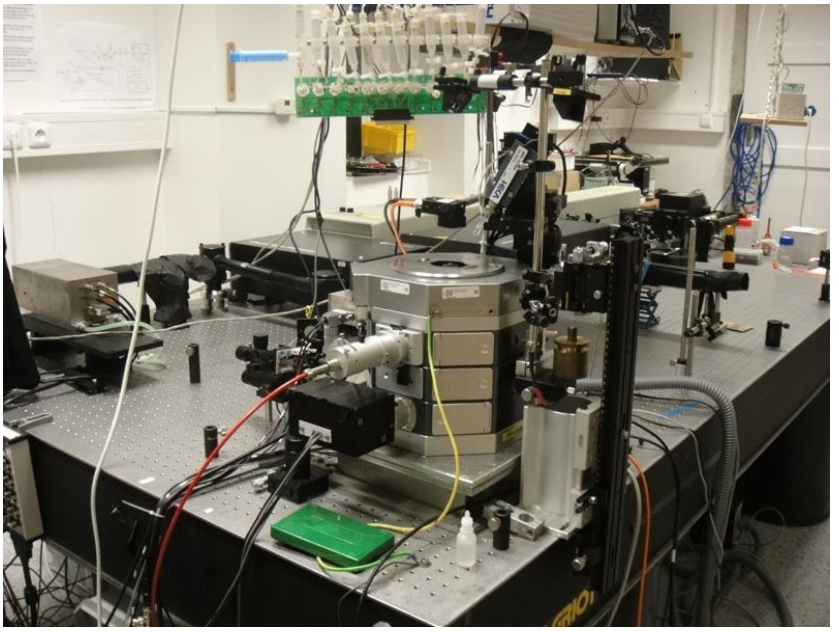

Figure 15. A photo of the 2PPM microscopy setup

2.3. Two-photon polarization microscopy (2PPM)

All 2PPM experiments were performed on an inverted iMic2 microscope (Figure 15) equipped with a Yanus scanhead (Till Photonics, Germany), 60x UApoPlan objective lens, NA 1.2 (Olympus, Japan) and a Chameleon Ultra II femtosecond pulsed infrared titanium:sapphire laser light source with Group Velocity Dispersion compensation unit (Coherent, USA). A scheme of our setup is shown in Figure 16. We used 850 nm light wavelength for eCFP excitation and 960 nm for eGFP and eYFP excitation. Laser output power was attenuated to 50 mW. Intrinsically linearly polarized laser light of Chameleon Ultra II laser (horizontal polarization > 500:1 (111)) was further purified with a polarizing beamsplitter before entering a polarization modulator, which allowed acquisition of images of fluorescently labeled cells with both horizontal and vertical liner polarizations of the excitation light. In initial experiments, a manually rotatable 690-1200 nm achromatic half-wave plate (Thorlabs, USA) was used for modulation of excitation light polarization between acquisition of individual images. To improve temporal resolution and relieve artifacts stemming from beam and cell movements, we have employed rapid electro-optical polarization modulation for our later experiments. An electro-optical polarization modulator (RPM-2P, Innovative Bioimaging, USA), was

synchronized with the microscope to allow alternating light polarization on a submicrosecond timescale, between acquisition of subsequent individual pixels. This way, the two images with distinct excitation light polarizations could be acquired virtually simultaneously. We typically used 10 μs pixel dwell time for image acquisition. After passing through the polarization modulator, excitation light was scanned by the microscope scanner and delivered to the sample by the objective lens. Epifluorescence was separated from the excitation light by a dichroic beamsplitter (Q565LP for eCFP and 740DCXR for eGFP and eYFP, both Chroma, USA) and filtered by an emission filter (479/40 nm for eCFP, 520/35 nm for eGFP and 542/27 nm for eYFP, all Semrock, USA). Fluorescence was detected by a PMT (R6357, Hamamatsu Photonics, Japan) equipped with an IR-blocking filter (HQ700SP-2P, Chroma, USA). Images were typically acquired at 200x200 nm pixel size when using the half-wave plate or 100x200 nm pixel size when using electro-optical polarization modulation (producing 200x200 nm images after de-convolution).

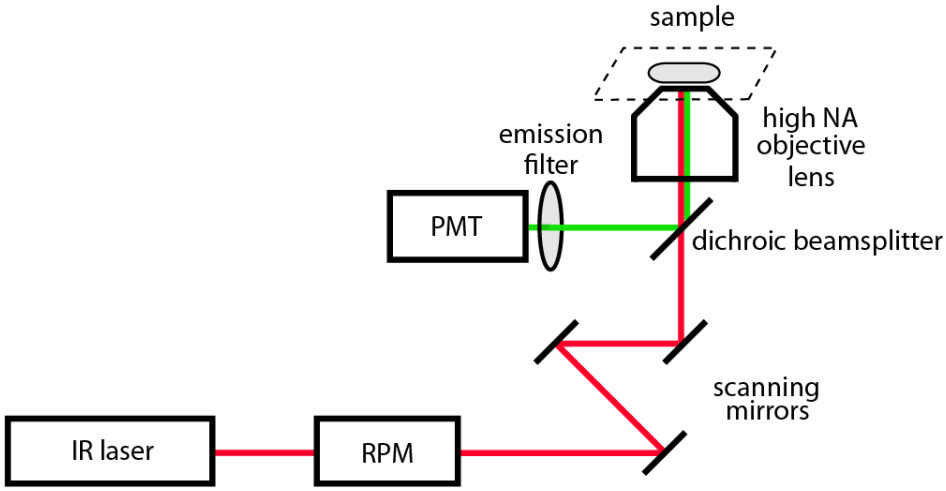

Figure 16. Schematic of the 2PPM setup. A 2PPM setup is similar to a common 2P microscopy setup, with the addition of a polarization modulator (RPM).

2.4. Image processing

Ratiometric imaging analysis was performed using ImageJ and Polaris+ (Innovative Bioimaging, USA) software. Images acquired using electro-optical polarization modulation were first de-interleaved into two separate images containing pixels corresponding to horizontal and vertical excitation light

polarization, respectively. Images were background subtracted and corrected for unequal intensity and pulse length of horizontally and vertically polarized excitation light. A ratiometric image was created from the corrected images by applying a lookup table designed to show fluorescence intensity by brightness and the dichroic ratio by color hue. Fluorescence emitted upon excitation with horizontal polarization was colored red and with vertical polarization green. Pure red and green colors indicate pixels with the dichroic ratio exceeding a chosen maximum value. Image processing steps are illustrated in Figure 17.

Quantitative processing of images was performed using a combination of available software (ImageJ, Icy) and in-house developed Matlab scripts. Original images were de-interleaved and background subtracted. A ratio image was created from obtained images and a mask was applied to this image in order to eliminate pixels corresponding to extracellular regions. Cell outline was manually selected and its shape approximated by a spline function (ImageJ) or a snake (Icy). Values of the dichroic ratio (r) were obtained separately for all pixels of the cell outline and $\log_2(r)$ of each value was calculated. Cell membrane orientation (angle θ) was determined for each pixel from a corresponding part of the spline or snake shape function, and pixels corresponding to horizontal ($\theta = \pi/2 \pm 3^o$) or vertical ($\theta = 0 \pm 3^o$) parts of the cell outline were used for determination of the maximal dichroic ratio (r_{max}). The r values were corrected for unequal intensity of horizontally and vertically polarized light by using the pixels corresponding to $\theta = \pi/4 \pm 3^o$, in which the mean r value should be 1.00 (and $\log_2(r) = 0$). We calculated the r_{max} by taking the mean value of r and $\log_2(r)$ for pixels corresponding to horizontally oriented parts of the cell outline and $1/r$ (and $\log_2(1/r)$) values for pixels corresponding to vertically oriented parts of the cell outline. Values of $r_{max} > 1$ (and $\log_2(r_{max}) > 0$) indicate that the mean angle of the 2P TDM of the fluorophore with respect to the cell membrane normal is larger than the 2P magic angle (52.0°) and that the 2P TDM is close to parallel to the cell membrane plane. Values of $r_{max} < 1$ (and $\log_2(r_{max}) < 0$) indicate that the fluorophore's 2P TDM is close to perpendicular to the cell membrane plane.

All results are quantitatively presented as mean±SEM. Statistical significance was tested using Student's t-test. Normality of all data was tested and confirmed by D'Agostino-Pearson k^2 Omnibus normality test.

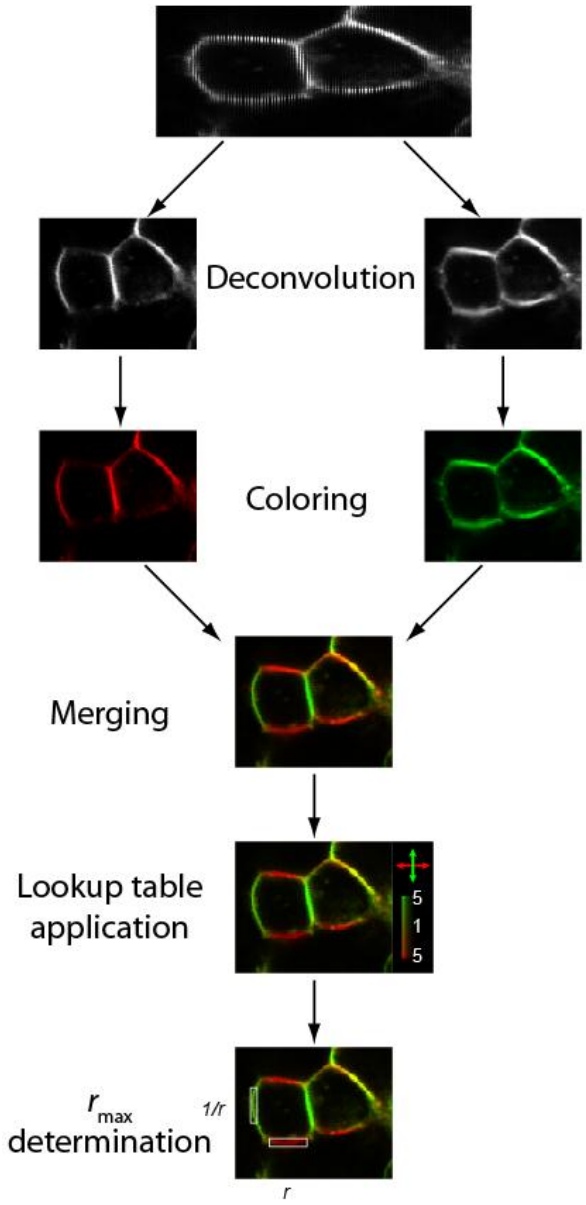

Figure 17. 2PPM image processing steps. The initial image is separated into two images containing odd and even pixels, respectively. The images are then corrected for enequal intensities of horizontally and vertically polarized laser light. The image containing odd pixels (corresponding to horizontal polarization of the excitation light) is colored red, and the image containing even pixels (corresponding to horizontal polarization of the excitation light) green. A lookup table is applied to show the values of r by the color hue. Horizontal and vertical parts of the cell outline are used for quantitative image processing and r_{max} determination.

The technique of 2PPM, including its theoretical background, experimental setup and data analysis, is described in Chapters 3.1 and 3.2.

2.5. Confocal polarization microscopy

The experiments were carried out using the same iMic2 microscope as described for 2PPM, but equipped with 405 nm and 488 nm diode lasers (Cobolt, Sweden) as light sources and 60x UApoPlan objective lens, NA 1.2 (Olympus, Japan). The RPM-2P (Innovative Bioimaging, USA) was used for light polarization modulation. Excitation light was separated from emitted fluorescence using a 458 long-pass dichroic beamsplitter and a 479/40 emission filter for CFP, and a 509 long-pass dichroic beamsplitter for eGFP and eYFP, and a 520/35 or 542/27 emission filter (all Semrock, USA), respectively. Fluorescence was detected using a PMT (R6357, Hamamatsu Photonics, Japan) in a de-scanned way. A confocal pinhole assembly for spatial signal filtering was mounted in front of the PMT. Image processing was similar to that described for 2PPM.

2.6. Förster Resonance Energy Transfer measurements

FRET experiments were performed on the same inverted iMic2 microscope using the same 60x Olympus objective lens as described for 2PPM. The microscope was equipped with a Polychrome IV light source (Till Photonics, Germany) and an ImagoQE CCD camera detector (PCO, Germany). The Polychrome IV emission was set to 438 nm and filtered through a 438/24 nm excitation filter. and reflected onto the sample by a 458 long-pass dichroic beamsplitter (both Semrock, USA). CFP fluorescence was filtered through a 472/30 nm emission filter, and eYFP fluorescence was filtered through a 542/27 nm emission filter (both Semrock, USA) mounted in front of the camera. For direct eYFP excitation we set the Polychrome IV to 488 nm and used a 479/40 excitation filter, a 509 long pass dichroic beamsplitter and a 542/27 emission filter (all Semrock, USA). The camera exposure time was set to 500 ms. Images were acquired sequentially. FRET was quantitated using the ratio of CFP to YFP fluorescence intensity (F_{CFP}/F_{YFP}). Acquired images were background subtracted and the FRET signal was corrected for CFP fluorescence bleedthrough. Direct YFP excitation was determined to be

negligible. We quantitatively analyzed at least 10 cells for each combination of constructs.

2.7. Agonist application

Initial experiments were carried out in 8-well microscopy μ-slides (Ibidi GmbH, Germany) and chemicals were applied by pipetting. In further experiments, 6-chamber perfusion μ-slides (Ibidi GmbH, Germany) were used, and continuous liquid flow was maintained using a peristaltic pump (Minipuls 3, Gilson, UK). The flow rate was usually set to 0.5-1.0 ml/min for G protein activation experiments and to 0.2 ml/min for Ca^{2+} concentration calibration experiments. Agonist application and washout times were determined by calibration of the system with a fluorescent dye (fluorescein isothiocyanate, Sigma, USA). Agonists were usually applied for 30 seconds, except for Ca^{2+} concentration calibration experiments, where ionomycin-containing solutions of known Ca^{2+} concentration were applied for 30 minutes to ensure equilibration of extracellular and intracellular Ca^{2+} concentrations.

2.8. Electrophysiology

HEK293 cells were plated in 24-well plates and transfected using as described in the Mammalian Cell Culture section. The day after transfection cells were trypsinized and transferred onto glass coverslips coated with poly-l-lysine. Pertussis toxin (Sigma, USA) was applied at concentration of 100 ng/ml 16 hours before electrophysiology experiments. Prior to electrophysiology experiments, DMEM was removed, cells were washed twice with PBS, the coverslips were transferred into an imaging chamber and an external solution (120 mM NaCl, 20 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.3) was applied. Patch pipettes with a resistance 2-6 MOhm were prepared from borosilicate glass capillaries (GC150T-10, Harvard Aparatus, USA) using a vertical puller PC-10 (Narishige, Japan) and filled with the internal (pipette) solution (100 mM potassium aspartate, 40 mM KCl, 5 mM MgATP, 10 mM HEPES, 5 mM NaCl, 2 mM EGTA, 1 mM $MgCl_2$, pH 7.3). Only individual cells well separated from others were used for measurements. Inward current recordings were performed using the whole cell patch-clamp technique (112) at a fixed holding potential (-90 mV) with an EPC10 USB amplifier and

Patchmaster software (HEKA Elektronik Dr. Schulze GmbH, Germany). Measured currents were normalized by cell capacitance (pA/pF). Normality of result distributions was tested by D'Agostino & Pearson k^2 Omnibus normality test. Final values were expressed as mean ± SEM and Student's t-test was used for evaluation of statistical significance.

2.9. Western blotting

HEK 293 cells were grown in cell culture 6-well plates and transfected (as described in the Mammalian cell culture and transient transfection section) 48 hours prior to lysate preparation. Transfection efficiency for each sample was calculated as the ratio of fluorescent cells to the total number of cells in at least 10 fields of view. Whole cell lysates were prepared using the RIPA buffer (Sigma) supplemented with cOmplete protease inhibitor (Roche), according to manufacturer's instructions. Samples were centrifuged at 10000 g for 5 minutes; supernatants were aliquoted and stored at -80 ^{o}C. Equal amounts of protein were used for polyacrylamide gel electrophoresis (Tris-Glycine, 12% polyacrylamide). After electrophoresis, gels were washed with water and soaked in the blotting buffer (Tris-glycine buffer with 20% methanol) for 10 minutes. Protein transfer onto 0.45 μm nitrocellulose membrane (or 0.2 μm membrane for Gγ2 subunit) was performed by electroblotting using the SE300 miniVE blotter (Hoefer, Germany) at 25 V for 90 minutes. Membranes were washed with Tris washing buffer containing Tween-20 (Sigma), and left in the blocking buffer (containing 5% non-fat dry milk) at constant shaking overnight. The membrane was then washed and incubated in an antibody buffer containing a primary antibody (1:200) for 2 hours, then washed 4 times with the washing buffer and incubated with the peroxidase-coupled secondary antibody (1:5000) for 1 hour and room temperature. The membrane was washed 4 times again and incubated with the TMB substrate (Sigma, USA) according to manufacturer's instructions. β-tubulin was used as a loading control. The following antibodies were used in experiments: polyclonal anti-Gαi1 (sc-391), anti-Gαo (sc-387), anti-Gβ1 (sc-379), anti-Gγ2 (sc-374), anti-β-tubulin (sc-9104), and goat anti-rabbit IgG-HRP (sc-2004) (all from Santa Cruz Biotechnologies, USA). Developed membranes were scanned to produce their digital images. The images were analyzed using Image Studio Lite software (Licor, USA).

3. Results and discussion

3.1. Two-photon polarization microscopy reveals protein structure and function

This chapter is based on Paper I:

Josef Lazar, **Alexey Bondar**, Stepan Timr and Stuart Firestein. **(2011)** Two-photon polarization microscopy reveals protein structure and function. *Nature Methods,* 8, 684-690.

ABSTRACT

Membrane proteins are a large, diverse group of proteins, serving a multitude of cellular functions. They are difficult to study, due to their requirement of a lipid membrane for function. Here we show that two-photon polarization microscopy can take advantage of the cell membrane requirement to yield insights into membrane protein structure and function, in living cells and organisms. The technique allows sensitive imaging of G-protein activation, changes in intracellular calcium concentration, and other processes, and is not limited to membrane proteins. Conveniently, many suitable probes for two-photon polarization microscopy already exist.

3.2. Dissociated $G\alpha_{GTP}$ and $G\beta\gamma$ protein subunits are the major activated form of heterotrimeric Gi/o proteins

This chapter is based on Paper II:

Alexey Bondar, Josef Lazar. **(2013)** Dissociated $G\alpha_{GTP}$ and $G\beta\gamma$ protein subunits are the major activated form of heterotrimeric Gi/o proteins. *Journal of Biological Chemistry*, 289, 1271-1281.

ABSTRACT

While most heterotrimeric G proteins are thought to dissociate into Gα and Gβγ subunits upon activation, the evidence in the Gi/o family has long been inconsistent and contradictory. The Gi/o protein family mediates inhibition of cAMP production and regulates activity of ion channels. Based on experimental evidence, both heterotrimer dissociation and rearrangement have been postulated as crucial steps of Gi/o protein activation and signal transduction. We have now investigated the process of Gi/o activation in living cells, directly by two-photon polarization microscopy and indirectly by observations of G protein-coupled receptor kinase-derived polypeptides. Our observations of existing fluorescently labeled and non-modified Gαi/o constructs indicate that the molecular mechanism of Gαi/o activation is affected by the presence and localization of the fluorescent label. All investigated non-labeled, non-modified Gi/o complexes extensively dissociate upon activation. The dissociated subunits can activate downstream effectors, and are thus likely to be the major activated Gi/o form. Constructs of Gαi/o subunits fluorescently labeled at the N-terminus (GAP43-CFP-Gαi/o) seem to faithfully reproduce behavior of the non-modified Gαi/o subunits. Gαi constructs labeled within the helical domain (Gαi-L91-YFP) largely do not dissociate upon activation, yet still activate downstream effectors, suggesting that the dissociation seen in non-modified Gαi/o proteins is not required for downstream signaling. Our results appear to reconcile disparate published data and settle a long-running dispute.

3.3. Gi/o proteins do not precouple to members of the Class A and C of G protein-coupled receptors

This chapter is based on Paper III:

Alexey Bondar, Josef Lazar Gi/o proteins do not precouple to members of the Class A and C of G protein-coupled receptors. *Manuscript*.

ABSTRACT

The Gi/o protein family transduces signals from a diverse group of G protein-coupled receptors (GPCRs). The observed specificity of Gi/o – GPCR coupling and high rate of Gi/o signal transduction have been proposed to be enabled by existence of stable associates between Gi/o proteins and their cognate GPCRs in the inactive state (Gi/o – GPCR precoupling). We have now applied the recently developed technique of two-photon polarization microscopy (2PPM) to two groups of Gαi/o subunits labeled with fluorescent proteins and two GPCRs (the α2A-adrenergic receptor (α2A-AR) and γ-aminobutyric acid receptor B ($GABA_B$)), to observations of Gi/o – GPCR interactions. Our experiments with non-dissociating mutants of a fluorescently labeled Gαi1 subunit (exhibiting impaired dissociation from activated GPCRs) show that 2PPM is capable of detecting GPCR – G protein interactions. However, no interactions between these non-dissociating mutants and the GPCRs can be observed prior to receptor activation. Results of 2PPM experiments with non-mutated fluorescently labeled Gαi/o subunits co-expressed with or without Gβ1 and Gγ2 subunits do not reveal any interaction of these proteins with non-stimulated α2A-AR or $GABA_B$ receptors. Therefore, our experiments provide evidence against stable physical interaction between Gi/o proteins and Class A and C GPCRs.

4. Conclusions

2PPM – a novel technique for studies of membrane protein structure and function

The initial aim of our research was to develop a microscopy technique for studies of membrane proteins which would take advantage of their membrane localization. Initial mathematical modeling results had suggested that using linear dichroism (unlike fluorescence polarization), particularly in combination with two-photon excitation, should allow sensitive observations of changes in structure of fluorescently labeled membrane proteins. In order to test this idea we developed the technique of 2PPM, which utilizes anisotropic properties of photon absorption by FP molecules (FP linear dichroism).

2PPM allows observations of linear dichroism (fluorescence detected) and of changes in this linear dichroism. Since linear dichroism is an effect of a bias in molecular orientations, 2PPM allows observations of changes in orientation of fluorescent molecules, such as FPs attached to membrane proteins. As functional activity of proteins is generally accompanied by changes in protein-protein interactions or in conformation that may affect the molecular orientation of a fluorescent moiety, 2PPM can be used for imaging of functional activity of membrane proteins, yielding both structural and functional information on the protein of study. We have successfully tested the ability of 2PPM to detect linear dichroism in FP-labeled membrane proteins, and used 2PPM to observe protein-protein interactions as well as changes in protein conformation in living cells.

We have shown that 2PPM can detect interactions between protein molecules, by observing interactions between G protein subunits. We were able to distinguish between free Gαi/o subunits and G protein heterotrimers by distinct values of LD in FP-labeled Gαi/o subunits (expressed alone and co-expressed with Gβγ dimers). Moreover, we have demonstrated that 2PPM allows sensitive detection of G protein activation in living cells in real time by observing changes in LD of the Gαi/o-FP constructs upon activation. The observed changes in LD were considerably higher than changes in FRET and BRET observed in previous studies (92,98,99,113) although the constructs we used had been specifically developed for FRET measurements and not for 2PPM measurements. The requirement for only a single fluorescent label, the pronounced changes in LD, and the considerable room for construct

improvements make 2PPM a valuable technique for studies of G protein activation and, in general, protein-protein interactions in live cells.

Apart from detecting protein-protein interactions, 2PPM should also allow detection of changes in membrane protein conformation in live cells. In order to test this, we tried to observe the changes in conformation of a FRET-based genetically encoded Ca^{2+} sensor termed LynD3cpV. We have found that 2PPM is able to detect changes in orientation of one of the two FPs (circularly permuted Venus, or cpV) present in LynD3cpV upon an increase of cytoplasmic Ca^{2+} concentration. Moreover, our methodology allows correlating changes of LD with changes in FRET and with absolute values of cytoplasmic Ca^{2+} concentration. These findings show that 2PPM can be used to detect changes in protein conformation, and that 2PPM data can be interpreted quantitatively, in terms of biologically relevant phenomena (such as concentration).

2PPM should be able to yield quantitative structural information about the protein of interest based on changes in orientation of its fluorescent label. We have developed a method for quantifying LD, which allows tentative determination of the mean tilt angle and variability of the 2P TDM with respect to the cell membrane. Once more data on 2P absorptivity tensors becomes available, and our method of structural interpretation of 2PPM data is verified by independent means, 2PPM should become capable of providing quantitative information on membrane protein structure.

The abilities of 2PPM to visualize molecular events and to provide structural insights allow 2PPM to be used not only for detecting a molecular event, but also uncovering its mechanism. We have shown this on the molecular processes of G protein activation. 2PPM has also brought insights into the mechanism of action of the genetically-encoded voltage sensitive protein Arclight A (Jin et al, manuscript).

2PPM possesses several advantages over existing microscopy techniques for studies of membrane proteins. 2PPM requires only a single FP for detection of protein-protein interactions, while other techniques, such as FRET, require two fluorescent moieties. Since 2PPM needs only a single FP tag and does not require heavy cell perturbation (such as membrane permeabilization or membrane protein cross-linking), it allows studying molecules and cells in

conditions closer to natural than previously possible. Because 2PPM relies on a single FP moiety (unlike, for example, FRET) to observe a molecular process, 2PPM allows easy simultaneous observations of multiple molecular processes (multiplexing). 2PPM relies on the full fluorescence signal obtained from the studied construct and changes in LD can be stronger than changes in FRET signal, even in probes developed for FRET. 2PPM provides both structural and functional information about the studied proteins. Using 2PPM one can determine not only changes in protein-protein interaction but also accompanying changes in protein conformation by directly observing changes in FP orientation. 2PPM data is intrinsically ratiometric and, therefore, independent of fluorescence intensity. 2PPM can be applied to many different fluorophores, both dyes and FPs. 2PPM works with many, if not all FPs derived from the original wtGFP from *A. Victoria*, as well as with FPs of the coral origin. 2PPM can yield information complementary to the data provided by other microscopy techniques. Also, many constructs suitable for 2PPM already exist and in many cases there is no need for development of new, 2PPM specific probes. All these features make 2PPM a technique of choice for studies of many membrane proteins in live cells.

The 2PPM technique has certain drawbacks and limitations. 2PPM can be utilized only for studies of proteins anchored to some oriented support, such as the cell membrane or cytoskeleton. 2PPM is sensitive to cell membrane orientation in the imaging plane. Therefore, cells with long stretches of horizontally and vertically oriented membranes are more suitable for 2PPM imaging than others. This limitation does not prevent measurements, but complicates finding suitable cells. Quantitative analysis of 2PPM images requires considerable image processing and a number of correction steps. Therefore, special software (developed by us and our collaborators) is required for efficient data analysis. Absence of LD both when the fluorophore molecules are oriented randomly and when they are all at the “magic angle” with respect to the cell membrane prevents distinguishing between the two cases. This drawback, however, could be overcome by using more than two excitation polarizations (two linear and one circular), or by combining 2PPM and single-photon confocal polarization microscopy. Development of these approaches is currently in progress. Temporal resolution of processes which can be detected

by 2PPM depends on the extent of change in FP orientation. Also, since 2PPM utilizes 2P excitation, all drawbacks of the 2P microscopy, such as low amounts of the total fluorescence signal, fast (albeit highly localized) bleaching, sample heating, and expensive equipment, apply to 2PPM as well. Although these limitations are unlikely to preclude successful application of 2PPM, they do constrain 2PPM's capabilities.

Can 1P excitation be utilized instead of 2P excitation to obtain similar results? We have investigated two possible 1PPM alternatives to 2PPM, namely, wide-field polarization microscopy and laser scanning confocal polarization microscopy. Both techniques could provide a relatively inexpensive and accessible alternative to 2PPM. However, in our hands, wide-field polarization fluorescence microscopy with excitation polarization modulation suffers from inhomogeneities in both polarization quality (partly due to scattering by the samples) and illumination intensities across the field of view. Furthermore, out-of-focus fluorescence from sections of the cell membrane parallel to the focal plane, lacking LD, tends to obscure the LD present in the cell outline.

1P confocal polarization microscopy is a much more promising alternative to 2PPM due to elimination of both out-of-focus fluorescence and fluorescence excited by scattered excitation light. Although sensitivity of confocal polarization microscopy to molecular orientation is smaller than that of 2PPM (due to $\cos^2\theta$ relation instead of $\cos^4\theta$) our results suggest that it allows detection of the majority of processes which can be studied by 2PPM. However, implementation of confocal 1PPM is not trivial, due to its stringent requirements for proper alignment (even more so with 1PPM than with regular confocal microscopy) and the fact that commercial confocal microscopes use optical fibers to carry excitation light.

Dissociated $G\alpha_{GTP}$ and $G\beta\gamma$ protein subunits are the major activated form of G proteins

The second aim of our research was to answer the long-standing question of whether the activated form of heterotrimeric Gi/o proteins is a re-arranged heterotrimer or the individual $G\alpha i/o_{GTP}$ and $G\beta\gamma$ subunits. This question has been widely disputed, with some studies suggesting dissociation of activated

Gi/o protein heterotrimers (33,99,114) and others suggesting their re-arrangement (82,92,95,98). We have applied 2PPM, along with several other experimental techniques in order to address this question.

We carried out 2PPM experiments on FP-tagged Gαi/o constructs and GRK3ct translocation measurements both on FP-tagged and untagged Gαi/o constructs. The results of both our 2PPM measurements and GRK3ct translocation observations show that fluorescently labeled Gi/o protein heterotrimers dissociate or re-arrange upon activation depending on the FP insertion site. We show that all the GAP43-CFP-Gαi/o constructs and the Gαo-L91-YFP construct, but not the Gαi-L91-YFP constructs dissociate upon activation. Our GRK3ct translocation studies on non-modified Gi/o proteins show that they all extensively dissociate into a free $G\alpha i/o_{GTP}$ subunit and a Gβγ dimer upon activation. Extrapolation of our data to endogenous protein expression levels indicates that activation leads to extensive (>85 %) dissociation of native Gi/o protein heterotrimers. We also demonstrate that dissociated G protein subunits are capable of stimulating effector molecules (GIRK channels). These results suggest that dissociated $G\alpha i/o_{GTP}$ and Gβγ subunits represent the major activated form of Gi/o proteins.

Our data show that the previously described selectivity of activation of GIRK channels, by Gβγ dimers arising from activated Gi/o proteins but not from other G proteins (115,116), cannot be explained by Gi/o protein heterotrimer rearrangement upon activation. Rearrangement in Gi/o protein heterotrimers, as opposed to dissociation in other types of G proteins, has been proposed (92) to explain the selective activation of GIRK channels by Gβγ dimers arising only from Gi/o proteins. However, our data demonstrate extensive dissociation of Gi/o protein heterotrimers upon activation and contradict this model. Our data are in line with an opposing model (93) of such selective activation, by free Gβγ dimers dissociating during activation from Gi/o heterotrimers, but not from other G proteins (as those undergo rearrangement, but not dissociation). Another plausible explanation for such selectivity is precoupling of inactive Gi/o proteins to GIRK channels (91).

The Gi/o protein association status upon activation affects localization of the individual G protein subunits, and therefore their interactions with effectors. Physical dissociation slows down Gi/o protein de-activation dynamics due to

spatial separation of the interacting proteins. Additional proteins (such as GRKs (86)) may be required for for timely re-association of free Gα subunits and Gβγ dimers into heterotrimers. Dissociated, activated G protein subunits have even been proposed to localize to distinct plasma membrane microdomains, because the palmitoyl and myristoyl tags of Gα subunit favor cholesterol-rich regions (such as lipid rafts); while the isoprenyl tag of the Gβγ dimer is preferentially excluded from such compartments (71). G protein effectors are known to be unevenly distributed in the cellular plasma membrane (71), and may be confined to membrane microdomains that host only particular types of G protein subunits. Spatial separation of G protein subunits upon activation might thus be required for selective modulation of effector activity as well as for control of heterotrimer re-association dynamics.

G protein effectors are capable of interacting with activated, non-dissociated G protein heterotrimers. A surprising finding for us was the ability of G proteins containing the Gαi1-L91-YFP construct (or its constitutively active mutant) to activate GIRK channels despite no detectable G protein heterotrimer dissociation. This result indicates that heterotrimer dissociation, although dominant in native Gi/o constructs, might not be strictly required for downstream signaling and that, at least in FP-labeled systems, $G\alpha_{GTP}G\beta\gamma$-effector complexes are likely to exist. It is possible that heterotrimer dissociation occurs simultaneously or after interaction with effectors. However, due to the rapid dissociation of activated non-modified Gi/o heterotrimers, the extent of downstream signaling by activated heterotrimers is likely limited.

Based on our results we conclude that Gi/o proteins extensively dissociate upon activation into free $G\alpha i/o_{GTP}$ subunits and Gβγ dimers. Free $G\alpha i/o_{GTP}$ and Gβγ represent the major activated form of Gi/o proteins. Individual $G\alpha i/o_{GTP}$ subunits and Gβγ dimers are capable of interaction with downstream effectors and regulation of their functional activity.

Gi/o proteins do not precouple to GPCRs in the inactive state

The third aim of our research was to determine whether Gi/o proteins physically interact with their cognate GPCRs in the inactive state. Interactions between GPCRs and G proteins determine the specificity and rate of signal transduction of the signaling cascade. It has remained unclear what factors

enable the fast rate of the specific interactions between an activated GPCR and G protein heterotrimers (117). It has been proposed that GPCRs form stable complexes (precouple) with G proteins in the inactive state (62). However, multiple studies of the coupling mode in live cells yielded inconsistent results (102,103,105). 2PPM, a new approach, offered to yield new insights into the mode of GPCR-G protein interactions. Since 2PPM can detect protein-protein interactions through using a single FP tag, 2PPM should allow obtaining information on GPCR – G protein interactions under conditions close to natural.

We have applied 2PPM to determine if Gi/o proteins precouple to two of their cognate GPCRs: the α2A-AR and $GABA_B$ receptors. Results of our 2PPM experiments with non-dissociating mutants of G proteins (103,118), forming stable complexes with activated GPCRs, demonstrate that interactions between the studied GPCRs and G proteins can be detected by 2PPM. However, our experiments with non-mutated constructs did not reveal significant interactions of individual Gαi/o subunits or Gi/o protein heterotrimers with the two investigated GPCRs.

Our results are not consistent with the GPCR – G protein precoupling model, and suggest that the α2A-AR and $GABA_B$ receptors interact with Gi/o proteins only transiently, upon stimulation of GPCR molecules with agonists, as postulated by the collision coupling model. In connection with the known fast rate (tens of milliseconds (117)) of GPCR-G protein interactions, our results argue for presence of mechanisms ensuring fast and efficient GPCR signal transduction other than physical GPCR – G protein precoupling, such as kinetic scaffolding (119) or spatial confinement of molecules to membrane microdomains (108, 120). Since the α2A-AR and $GABA_B$ receptors we investigated belong to distinct GPCR classes (Class A and C, respectively), our results are likely valid for a wide range of GPCRs that couple to the Gi/o proteins, although other classes of G proteins and GPCRs may still precouple. However, based on our 2PPM observations we conclude that non-stimulated Class A and Class C GPCRs do not form stable complexes with Gi/o proteins, and that the known specificity and spatiotemporal dynamics of Gi/o protein interactions with activated GPCRs are the result of other molecular mechanisms.

5. References

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