**Working title: Visualizing G protein-coupled receptor homomers using** **photoactivatable dye localization microscopy**

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1. **Abstract**

G protein-coupled receptor (GPCR) di/oligomerization has revealed potential mechanisms for receptors diversification of signal selectivity, specificity, and amplitude. The use of super-resolution imaging techniques to investigate these di/oligomer molecular complexities have undoubtably provided insight to the dynamics of complexes formed at the plasma membrane. Here we describe the methodology of photoactivatable dye localization microscopy (PD-PALM) to study the spatial organization of GPCR homomers at the plasma membrane.

1. **Introduction** 
   1. ***Techniques used to study GPCRs***

G protein-coupled receptors (GPCR) are the largest and most diverse superfamily of membrane protein receptors. As a result of their necessity in mediating many physiological processes, they are the target of ~40% of prescription drugs (Sriram & Insel, 2018; Farran, 2017; Santos et al., 2017; Rask-Andersen, Masuram, & Schiöth, 2014), therefore discoveries into understanding the mechanisms governing their functions remain significant. Due to their dynamic nature and functional and signal pleiotropy, GPCRs remain amongst the least understood family of membrane receptors. Recent advances and breakthroughs in the field have unveiled new insights into multiple structural facets of GPCRs (Gusach et al., 2020), their interactions with other proteins (Gurevich & Gurevich, 2019) and their dynamics (Wingler & Lefkowitz, 2020). Yet, we still have limited understanding on what controls GPCR function, particularly in native cells.

Some of the earliest studies using biochemical approaches revealed that GPCRs can form dimers and/or oligomers as a potential way of modulating GPCR trafficking, ligand specificity, and signal selectivity and amplitude (Wang, Qiao, & Li, 2018). Structural techniques, such as x-ray crystallography and cryo-electron microscopy (cryo-EM), have unveiled the structures of many GPCRs di/oligomers in their active and inactive states (Mao et al., 2020; Shaye et al., 2020; Zhao et al., 2019; Warne et al., 2008; Rasmussen et al., 2007; Palczewski et al., 2000). These techniques, alongside convergent interdisciplinary approaches (Milligan, Ward, & Marsango, 2019), have been able to determine the interacting interfaces of GPCRs di/oligomers and have provided the foundation for initiating the therapeutic targeting of GPCR di/oligomers (Huang, St Onge, Ma, & Zhang, 2021; Grant et al., 2019; Farran, 2017; Yuan et al., 2012).

Biophysical approaches have advanced our understanding of the role of GPCR di/oligomers, uncovering structural properties and dimerization interfaces, and dynamics of interactions. Indeed, bioluminescence/fluorescence-based complementation assays have shed light on GPCR dimer interactions with either the same GPCRs (homomers) or with other membrane-bound proteins (heteromers) (Wouters, Vasudevan, Crans, Saini, & Stove, 2019). It has also unveiled the dynamics of GPCR interaction with other proteins like arrestin (Lee et al., 2016; Nuber et al., 2016; Zimmerman et al., 2012; Shukla et al., 2008) and G proteins (Wan et al., 2018; Ayoub et al., 2007; Galés et al., 2005; Bünemann, Frank, & Lohse, 2003). However, RET techniques are limited in sensitivity and resolution.

Single molecule imaging has been shown to be a powerful tool for the study of molecular interactions within the plasma membranes (Kasai, Ito, Awane, Fujiwara, & Kusumi, 2018; Calebiro et al., 2013; Kasai et al., 2011). The use of total internal reflection fluorescence microscopy (TIRF-M) for primarily imaging receptor-receptor interactions and the nature and lifetime of such interactions, has further advanced the field (Kasai et al., 2018; Sungkaworn et al., 2017; Tabor et al., 2016; Calebiro et al., 2013; Kasai et al., 2011; Hern et al., 2010). Likewise, spatial intensity distribution analysis (SpIDA) has investigated GPCR-GPCR dynamics in live cells, providing temporal resolution (Pediani, Ward, Marsango, & Milligan, 2018; Marsango et al., 2017; Godin et al., 2015; Ward, Pediani, Godin, & Milligan, 2015). However, the limitation is that these techniques are diffraction-limited and therefore require low receptor expression (1-10 molecules/μm2), to resolve distinct point spread functions of individual receptors. This poses problems for understanding the potential physiological implications of such receptor-receptor interactions, where receptor expression is higher than this in native cells.

Super-resolution imaging approaches provide a means to overcome the diffraction limits of standard microscopy and issues with receptor expression levels. As such, super-resolution imaging approaches such as stochastic optical reconstruction microscopy (STORM), stimulation emission depletion (STED) microscopy, and photoactivatable localization microscopy (PALM) provide promising avenues for uncovering aspects of GPCR functions, interactions and GPCR dynamics beyond the diffraction limit. To date, the only super resolution imaging technique that has been employed is PALM to investigate GPCR interactions (Jonas et al., 2018; Jonas, Fanelli, Huhtaniemi, & Hanyaloglu, 2015; Scarselli, Annibale, & Radenovic, 2012). PALM imaging breaks the diffraction limit barrier of conventional light microscope, typically offering a resolution of 10-20nm, and can investigate GPCR dynamic in cells expressing higher receptor density levels. The ability to stochastically activate and photobleaching of photoactivated fluorophores facilitate the spatially separate localization of individual receptor molecules. This enables the capture and localization of non-overlapped point spread function (PSF) from activated dyes/fluorophores, and resolution of individual GPCR protomers.

* 1. ***PD-PALM as a tool to study GPCR oligomerization***

The technique of photoactivated dye localization microscopy (PD-PALM) has been successfully developed and utilized to identify and localize single GPCR molecules on the plasma membrane of fixed cells. It has proven to be a very useful tool in aiding our understanding of gonadotropin hormone receptor homomers and heteromers. With these receptors playing essential roles in both male and female reproductive function, and dysfunction resulting in disorders causing sub/infertility (Dumesic et al., 2015; Huhtaniemi, 2006; Abel et al., 2000; Dierich et al., 1998), understanding the mechanism governing their functionality are of high importance in the assisted reproductive arena. Our previous work proposed and utilized PD-PALM to quantitate the number of luteinizing hormone receptor (LHR) oligomers at the plasma membrane and the percentage of each type of oligomeric complexes formed i.e., dimer, trimer, tetramer, etc (Jonas et al., 2015). This study revealed that approximately 40% of wild type LHR (expressed in heterologous cell lines) were associated as homomers with most of the complexes as dimers was expressed (Jonas et al., 2015). However, co-expression ligand binding-deficient LHR (LHRB-) and signal-deficient LHR (LHRS-) mutants, previously shown to functionally cooperate and undergo forced di/oligomerization (Rivero-Muller et al., 2010), favored the formation of oligomeric complexes over dimers (Jonas et al., 2015). Interestingly, ligand-dependent differences were observed in the ability to active Gαq/11-dependent IP3 and Ca2+ in the transactivating mutant LHRs. Moreover, further analysis revealed that the relative ratiometric composition of LHRB-:LHRS- within oligomeric complexes correlated with the degree of Gαs and Gαq/11 signaling observed, showing functional asymmetry within oligomers (Jonas et al., 2015).

Our previous work has also suggested potential roles of LHR/FSHR heteromers. During the peri-ovulatory phase of the ovarian cycle, LHR and follicle stimulating hormone receptor (FSHR) have been shown to be co-expressed in the granulosa cell compartments. However, the functional role remained elusive. By employing PD-PALM combined with analysis of Gαs and Gαq/11-dependent signaling analysis, we determined that the LH-induced formation of asymmetric LHR-FSHR heterotetramers promoted a sustained calcium response, with a concomitant abrogation in cAMP signaling (Jonas et al., 2018). Although the physiological roles remain unknown, the role of LH-dependent Gαq/11 activation in mediating ovulation has been demonstrated in mouse models (Breen et al., 2013). It may be that the formation of LHR/FSHR heteromers aids and favors the propagation of G αq/11-dependent signaling.

As outlined, PD-PALM has proved a useful tool to investigate GPCR oligomerization. We will next describe the methodology for setting up PD-PALM experiments for analyzing GPCR homomers and provide a commentary of useful hints and tips throughout.

1. **Materials** 
   1. ***Plasmids***

An N-terminally HA-tagged FSHR (HA-FSHR) in pcDNA3.1 plasmid was constructed, as previously described (Jonas et al., 2018).

* 1. ***Cell lines and cell culture***

Immortalized human embryonic kidney cell line (HEK293) was obtained from ATCC. Cells were maintained and cultured at 37°C in 5% CO2 in Dulbecco′s Modified Eagle′s Media (DMEM, Sigma Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich) and 1% Antibiotic-Antimycotic (ThermoFisher).

* 1. ***Antibodies and CAGE fluorophore***

To identify and localize GPCRs, there needs to be an appropriate method for labelling receptors of interest. Photoswitchable proteins have been employed to image intracellular proteins (Chudakov, Matz, Lukyanov, & Lukyanov, 2010; Betzig et al., 2006). Other studies investigating the FSHR have utilized FSHR antibodies, however, they have been shown to be notoriously non-specific and can affect ligand binding and receptor signaling (Vannier, Loosfelt, Meduri, Pichon, & Milgrom, 1996).

For PD-PALM an antibody specific to the tagged-GPCR requires PD labelling. We utilized monoclonal HA.11 antibody (16B12, BioLegend) and labelled it with NHS-ester CAGETM 552 PD (Abberior). We have typically found that a 5-10 fold molar excess of PD:antibody yields a 1:1 stoichiometry. The stoichiometry of dye:antibody was calculated using the degree of labelling (DOL) calculation based on a derivation of the Beer-Lambert law (see Equation 1), which is explained in further detail in the manufacturer’s protocol (Abberior GMBH) for labelling.

Shape

Description automatically generated with medium confidence

**Equation 1.** The degree of labelling can be calculated using a derivation of the Beer-Lambert law. Amax = absorbance of the dye at maximum absorbance; A280 = absorbance of the dye at 280nm; εmax is the extinction coefficient of the dye at the absorbance maximum; ε280 is the extinction coefficient of the dye at 280nm; εProt is the extinction coefficient of the antibody at 280nm; AProt is the absorbance of the antibody at 280nm; C280 is the correction factor of the dye given by C280 = ε280 / εmax.

1. **A step-by-step guide to PD-PALM imaging and analysis**
   1. ***Plating cells and transient transfection***

Plate HEK293 cells into 6-well tissue culture-treated plates (~600,000 cells/well) and incubate overnight, at 37°C at 5% CO2 in air. The following day, select wells that are 70-80%[[1]](#footnote-1) confluent for transfection.

Next, transfect cells with the GPCR of interest. For our studies we utilize Lipofectamine 2000TM (Thermofisher). For Lipofectamine 2000 transfections, prepare transfection mixes as per manufacturer’s instructions, by aliquoting 250μl[[2]](#footnote-2) of low serum medium OptiMEM (Thermofisher) into two separate 1.5 ml microfuge tubes, labelled ‘Tube A’ and ‘Tube B’ (Figure 1A). To ‘Tube A’ add 8μl of Lipofectamine 2000TM and to ‘Tube B’ add 3 μg[[3]](#footnote-3) of HA-FSHR in pcDNA3.1 plasmid (or GPCR of choice) and incubate for 5 minutes at room temperature (RT). Following this, add the contents of ‘Tube B’ to ‘Tube A’, gently mix and allow this to sit for a further 20 minutes at RT so Lipofectamine 2000TM complexes with plasmid DNA. Once completed, add the entire Lipofectamine 2000TM-DNA complexes onto the plated cells in a drop-wise fashion and incubate for 48 hours at 37°C.

The choice of slide and slide thickness is an important consideration for super-resolution imaging to ensure the best quality images with optimal localization precision are obtained. For our studies we used 8-chamber wells 1.5 borosilicate cover glass slides, which has high quality thickness with minimal variation. A two-stage plating process is required such that 24 hours post-transfection cells are re-plated into 8-chamber well cover glass slides (Figure 1A). Effective and optimal TIRF imaging requires cells being distinct and in a monolayer. Since the doubling time of our HEK293 cell strain is ⁓36 hours, re-plating cells ensures approximately 50% confluency is achieved prior to treatment. Furthermore, this two-stage step is also necessary as it allows sufficient time for transcriptional/translational processing of GPCRs and trafficking to the cell surface, and so most studies carry out functional analysis 48-72 hours post-transfection.

* 1. ***Stimulation***
     1. *Cell treatment*

48 hours post-transfection, cells are first pre-incubated for 30 minutes with labelled anti-HA-CAGE 552 antibody (or labelled antibody and dye of your choice) (Figure 1B). From our experience of extensively testing blocking agents, we found that diluting the antibody (1/250) in DMEM supplemented with 10% FBS prevented non-specific binding of the HA.11 antibody as previously described (Jonas, Huhtaniemi, & Hanyaloglu, 2016). All steps hereon must be performed in light-protected conditions to prevent spontaneous uncaging of fluorophores.

Following pre-incubation, excess antibody should be carefully removed from individual chamber wells and discarded. Cells can then be treated with a ligand of choice for the required time. For our studies with the FSHR we typically treated cells with 0-100 ng/ml of FSH for up to 15 minutes. At the end of treatment, carefully remove the media and wash cells in PBS (3 x 5 minutes).

* + 1. *Fixation*

Fix cells in 4% PFA with 0.2% glutaraldehyde for 30 minutes at RT under light-protected conditions (Figure 1B). This combination of fixatives has been shown to prevent lateral diffusion of transmembrane proteins when compared to fixation with 4% PFA alone (Annibale, Vanni, Scarselli, Rothlisberger, & Radenovic, 2011; Tanaka et al., 2010). Wash cells in PBS (2 x 5 minutes) and store in fresh PBS. Fixed cells can then be stored in a light-controlled box at 4°C until imaged. We typically image labelled cells within 24 hours and up to a maximum of 72 hours post-labelling.

* 1. ***Imaging***
     1. *Live time-lapse microscopy*

To image the GPCR cell surface landscape, we utilize the Zeiss Elyra PS.1 super resolution microscope. This microscope can map single molecules of labelled proteins expressed at physiological levels (200 molecules/μm2) to a localization precision of ~20nm, breaking the diffraction barrier of conventional light microscopes that have a resolution of ⁓200 nm.

The microscope consists of a cooled electron multiplying charged-coupled device camera (EM-CCD; C9100-13, Hamamatsu) that enables the simultaneous imaging of two-color PD-PALM via two separate channels. It is equipped with 100x objective lens with a 1.45 numerical aperture (NA), resulting in better resolution and a perfect focus setup, to prevent Z-plane drift. In addition, it has multiple lasers lines (405 nm, 488 nm, 561 nm and 642 nm) depending on the fluorophore used. For set up, lasers are typically switched on 30 minutes prior to imaging to allow enough time to heat up and stabilize. This additionally facilitates the cooling of the EM-CCD and PALM camera to -70°C. The Zeiss Elyra PS.1 is enclosed in a dark box to prevent spontaneous uncaging of PDs and to aid the regulation of the internal surrounding temperature to about 25°C, as excess heat generated by the lasers can cause this to fluctuate. It is also mounted on an anti-vibration table to minimalize sample drift and vibrations during imaging. These features collectively conserve the stability and integrity of imaging and to ensure accurate localization precision of data.

* + 1. *Locating cells and visualizing GPCR molecules*

Before commencing imaging, the 100x 1.45 NA objective lens needs to be cleaned using 70% ethanol and allowed to fully evaporate before placing a drop of immersion oil with the correct refractive index for imaging cells immersed in PBS (Figure 1C). The chamber slides containing the fixed cells in PBS can now be placed onto the microscope stage. The brightfield setting is utilized to ensure a section of cells within the eye piece view are in the correct focal plane before switching over to fluorescence live mode via the locate tab on the ZEN software. Once switched over to fluorescent live mode, maintaining low laser power and without switching on the 405 nm laser to ensure minimal photoactivation, the brightness and contrast needs to be adjusted to visualize the selected region of cells. The histogram can be set between the minimum/maximum initially and then further adjusted to observe the cells while omitting background brightness/noise and optimizing signal/noise.

To visualize cell surface GPCRs, cells need to be imaged at an optimal TIRF-angle (Figure 1C). To first locate the plasma membrane, it is easier to find the coverslip by scrolling through the Z-plane. Once the coverslip has been located it becomes a case of achieving a fine balance between adjusting the Z-plane and the most acute TIRF angle until the cell surface is observed and fluorophores can be seen activating within the cell boarders, with minimal background noise.

* + 1. *PD uncaging and bleaching*

Once all the adjustments and focusing have been made, the imaging experiment of GPCRs in a selected region of interest (ROI) can begin. For our studies, we use a 24.9 x 24.9 μm ROI. Once imaging commences, the definite focus can be switched on to prevent sample drift during imaging.

For image acquisition, the image speed (frames/second) is something to consider when conducting the imaging experiments. Imaging too fast usually results in the same activated fluorophore being detected over multiple frames and can cause an overestimation of GPCR oligomers, whereas imaging too slow often results in missing activated fluorophores, thus causing an underestimation of GPCR oligomers. Time-lapsed image series need to be set up and taken over several frames during the imaging experiment. For our studies we typically image ~31,500 frames at 8 frames/second.

The laser power used will determine the number of fluorophores activated and hence the degree of spatial separation. To uncage fluorophores, the 405 nm UV laser is required, and for our studies using the CAGE 552 PD the 561 nm laser is required for photobleaching. Since it will also determine the efficiency of photobleaching, it is imperative that a fine balance is achieved between achieving spatially separated fluorophores and effective photobleaching. As a result, laser power is initially low and gradually increased during imaging experiments to ensure all fluorophores are photoactivated and sufficiently photobleached.

* 1. ***Resolving of GPCR molecules***
     1. *Determining GPCR x-y coordinates using QuickPALM*

The spatially separated detection of the activated PD molecules across the entire length of the imaging series creates a CZI compiled movie file. ImageJ (Fiji) software is then used to localize the single molecules from the acquired PD-PALM movies. The CZI files first need to be opened to ImageJ and the brightness and contrast adjusted to visualize the cells. Since there are minor adjustments made to the Z-plane and the TIRF angle at the beginning of imaging experiments, the frames containing these adjustments need to be removed from analysis. Following this, 5 x 5 μmnon-overlapping cropped sections from within the ROI are duplicated to perform post-acquisition analysis using QuickPALM; an ImageJ plugin to enable particle detection and image reconstruction (Figure 1D). To refine the stringency of the single molecules detected, for our studies with the FSHR and CAGE 552 PD we ensured that only fluorophores with a signal to noise ratio (SNR) ≥8 and a full-width, half-maximum (FWHM) ≤3, were determined by our imaging parameters. However, these parameters may need to be adjusted for other PDs and microscope set-ups used. As the QuickPALM analysis runs, the live feed of detected fluorophores across the full length of the frames are mapped onto a single excel spreadsheet that localizes the individual GPCR molecules to individual x-y coordinates on separate lines (Figure 1D).

* + 1. *Molecule filtering*

Due to the difficulty associated with finding the balance photoactivation and photobleaching, some molecules can stay ‘on’ for more than 1 frame, which could lead to an overestimation of GPCR oligomers. To prevent this, we have generated an algorithm to remove molecules that have persisted for >1 frame. This JAVA run program has been designed to read through the x-y excel coordinates generated by QuickPALM files line-by-line. It allows you to edit the number of lines it searches and the radius of the search area, such that it typically removes <1% of molecules. The parameters we filter are based on a search radius within the localization precision and we typically filter for molecules that have remained ‘on’ within 15 consecutive frames. However, these parameters would need to be adjusted based on individual microscope set up and localization precision of detecting individual GPCR activated molecule.

* + 1. *Quantitation of GPCR di/oligomers*

To determine the spatial organization of the detected GPCRs, and quantify the number of GPCRs existing as monomers, dimers and oligomers, we next employ a Getis-Franklin-based nearest neighborhood analysis. This analysis technique uses a single identified molecule and recursively searches within a given radius for any further molecules until no further molecules are located (Figure 1D). These GPCR molecules are then grouped together and the type of oligomer, e.g., trimer, tetramer, etc., are quantified.

For our studies we used a JAVA-based program, PD-interpreter, that was designed to carry out this analysis and can be freely download at [www.superimaging.org](http://www.superimaging.org). We typically set the search radius at 50 nm because the Class A glycoprotein hormone receptors, such as the FSHR, are known for their large extracellular domain and so are typically ⁓8 nm in size, coupled with the antibody-dye label size (⁓20 nm) and localization precision based on the PSF for the PDs (⁓20. nm) (Figure 1D). The size of other GPCRs, in addition to the antibody and PD used, would need to be carefully considered when selecting the best search radius.

Once the search radius is set, the analysis is run, and an output cluster file is generated. The data are represented as an image with overall plotted individual receptors based on the x-y coordinates, a heat map of GPCR homomers grouped into different oligomeric complexes and a separate excel spreadsheet quantifying the total number of resolved GPCR homomers, and the number of each GPCR homomeric subtype, mirroring the heat map (Figure 1D). This data can then be utilized to determine the number of non-associated GPCR molecules (monomers) and self-associated GPCR molecules (homomers), as well as the subtype of each GPCR homomeric complex (e.g., dimers, trimers, tetramers, pentamers, 6-8 oligomers or ≥9 oligomers) (Figure 1D).

1. **Future directions**

Advances in super-resolution imaging has provided for the first time the ability to determine GPCR spatial arrangements at a single-molecule level at the plasma membrane, enabling the study of GPCR homomeric and heteromeric complexes at receptor densities observed under physiological conditions. Our work to date has utilized PD-PALM to understand the functional roles of GPCR di/oligomers in fixed heterologous cells lines expressing receptors of interest. However, with current developments in new variants of fluorescent photoactivated proteins and photoactivatable ligands advancing (Ast et al., 2020; Gallo, 2020), developing ligands/genetic approaches to track GPCRs in fixed and live cells, provides exciting means to investigate GPCR homomerization/heteromerization in physiologically relevant cell types. The application *in vivo* would provide a framework to begin to understand the functional and physiological significance of GPCR homomers. Furthermore, utilization of labelled ligands will provide much needed information on GPCR ligand occupancy within different oligomeric complexes, and the role of ligand occupancy in directing monomer, dimer and oligomer formations and lifetimes. Future applications of PD-PALM where multichannel imaging can be applied will provide a means to uncover how individual GPCR complexes direct signaling. Current studies have implied super resolution imaging to assess trafficking of the GPR56 in pancreatic β-cells (Olaniru et al., 2021). Moreover, extending PD-PALM to image GPCR monomer, dimer and oligomer trafficking to and from the plasma membrane, and engagement with trafficking machinery, will begin to unravel whether different GPCR complexes have differential roles in receptor desensitization, internalization, and signaling.

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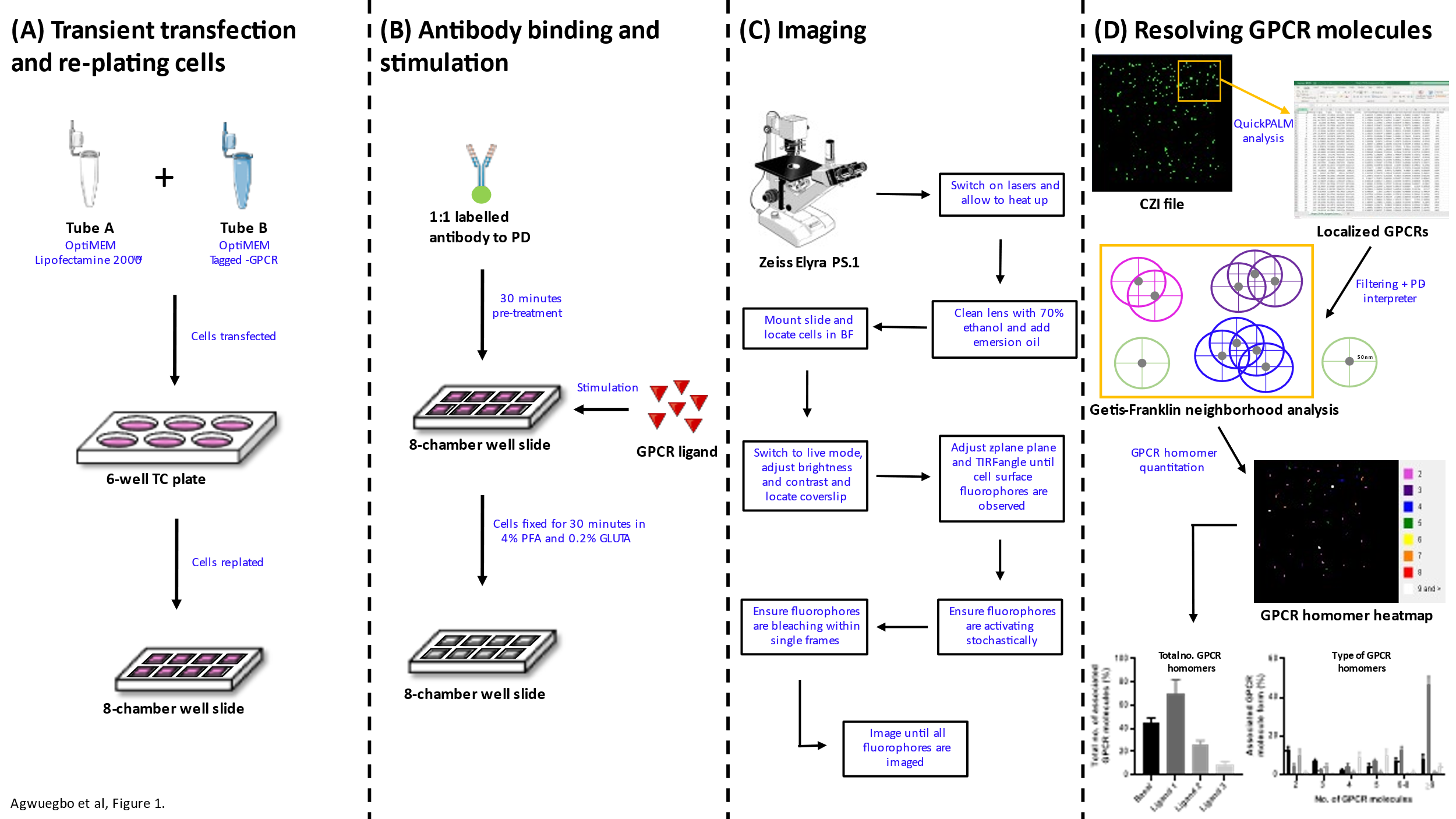
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**Figure Legends**

**Figure 1. Schematic flow diagram summarizing the steps required to visualize G protein-coupled receptor homomers using PD-PALM. (A)** Following the transient transfection of tagged-GPCR of interest into cells cultured in 6-well TC plates, re-plate cells into 8-chamber well slides. **(B)** Incubation cells for 30 minutes with a PD-labelled antibody complimentary to the GPCR tag. Stimulate cells with ligand/receptor modulator and post-treatment fix cells for 30 minutes. **(C)** Image cell surface PD-labelled GPCRs using the Zeiss Elyra PS.1 microscope. Following a series of set-up steps, all fluorophores need to be activated and bleached using specific laser lines within a single imaging experiment. **(D)** GPCR molecules identified during the imaging experiment are resolved, first using Fiji plug in, QuickPALM, to localize GPCR x-y coordinates. Coordinates are filtered and quantified using Getis-Franklin neighborhood analysis. This generates a GPCR homomer heat map that quantifies the total number and types of GPCR homomers complexes.



1. Using wells containing cells that are under- or over-confluent will compromise transfection efficiency. [↑](#footnote-ref-1)
2. Volume amounts specified are ideal for an individual well within a standard 6-well TC plate. [↑](#footnote-ref-2)
3. DNA amounts may vary depending on GPCR expression levels and transfection efficiency; therefore, optimization steps would need to be performed prior. [↑](#footnote-ref-3)