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A High-Content, Live-Cell, and Real-Time Approach to the Quantitation of Ligand-Induced β -Arrestin2 and Class A/Class B GPCR Mobilization

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Abstract

We report the development of a method to analyze receptor and β -arrestin2 mobilization between Class A and B GPCRs via time-resolved fluorescent microscopy coupled with semiautomated high-content multiparametric analysis. Using transiently expressed, tagged β_2 -adrenergic receptor (β_2 -AR) or parathyroid hormone receptor type 1 (PTH₁R), we quantified trafficking of the receptors along with the mobilization and colocalization of coexpressed tagged β -arrestin2. This classification system allows for exclusion of cells with nonoptimal characteristics and calculation of multiple morphological and spatial parameters including receptor endosome formation, β -arrestin mobilization, colocalization, areas, and shape. Stimulated Class A and B receptors demonstrate dramatically different patterns with regard to β -arrestin interactions. The method provides high kinetic resolution measurement of receptor translocation, which allows for the identification of the fleeting β -arrestin interaction found with β_2 -AR agonist stimulation, in contrast to stronger mobilization and receptor colocalization with agonist stimulation of the PTH₁R. Though especially appropriate for receptor kinetic studies, this method is generalizable to any dual fluorescence probe system in which quantification of object formation and movement is desired. These methodologies allow for quantitative, unbiased measurement of microscopy data and are further enhanced by providing real-time kinetics.

Keywords

high content; GPCR; adrenergic; parathyroid; arrestin; colocalization; kinetics; image processing; microscopy

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Supplementary Methods, Table S1, Files, and Movies

To view the Supplementary Methods, Table S1, Files, and Movies for this article, please visit <http://dx.doi.org/10.1017/S1431927612014067>.

Introduction

Microscopy and the quantitation of microscopic data are in a constant state of development. One of the remaining shortcomings is the inability to present quantitative data for an entire dataset. Classically, single images are presented as representative for a population, with quantitation of a sample of multiple images scored manually. Although representative images may in fact be reflective of accurate biology, the context of a single cell's response compared to a population is discarded. Manual scoring, counting, and classification methods, in addition to being low throughput, are also subject to significant sampling and observer bias. It is well known that even in an isogenic monoclonal cell line there is diversity and heterogeneity, and most cell cultures are even more complex and heterogeneous (Atwood et al., 2011). Further confounding imaging-based analysis is the variability of gene expression in transient transfections and lack of synchronization in a dividing culture. We now demonstrate an analysis method where dually transiently transfected HEK293 cells are identified, irrelevant cells are removed, and fluorescence protein dynamics are quantitated using object segmentation and multiparameter analysis for real-time fluorescence microscopy. Full dataset quantification coupled with error calculation provides confidence in determining the canonical ligand-mediated response in a given population of cells.

High content analyses of G protein-coupled receptor (GPCR) internalization have been instrumental in assays to identify compounds that can induce or alter GPCR-mediated signaling. Many have been used as an end-point fixed cell assay (Ghosh et al., 2000; Haasen et al., 2006; Ross et al., 2008; Fichter et al., 2010; Kwon et al., 2012). Highlighting the importance of this work, investigators have developed quantum dot labeling and electrical resistance/impedance sensors in addition to fluorescent-tagged constructs to study GPCR activation (Yu et al., 2006; Fichter et al., 2010; Stallaert et al., 2012). β -arrestin2 translocation is also an accepted and robust marker for the initiation of GPCR signaling (Ferguson & Caron, 2004).

Another set of elegant techniques include fluorescence and bioluminescence resonance energy transfer (FRET and BRET). TransfluorTM and other BRET-based assays, for example, have been used to characterize the recruitment of β -arrestin2 to membrane-bound GPCRs (Perroy et al., 2004; Hamdan et al., 2005; Ross et al., 2008; Kamal et al., 2009). However, these assays tend to measure whole well changes and do not analyze information on a cell-by-cell basis. These assays are also limited as the kinetic profiles of β -arrestin are not necessarily conserved between different classes of GPCRs or cell types. We report a robust assay that simultaneously measures the localization of fluorescently tagged receptor and β -arrestin granules in live cells at a high kinetic resolution (every 30 s) and determines colocalization by a subtractive method.

Early events in ligand mediated signal transduction are the basis for initializing cellular responses and quality quantitative data of these events are critical for understanding complex signaling events (Hanyaloglu & von Zastrow, 2008). As an experimental platform to validate object segmentation based high-content quantitation, we explored canonical ligand stimulated GPCR internalization and β -arrestin2 trafficking. Upon ligand mediated

activation, most GPCRs internalize and desensitize (Luttrell & Lefkowitz, 2002). Phosphorylation of the GPCR carboxy-terminus by G protein-coupled receptor kinases (GRKs) occurs following receptor activation, which enhances nonvisual arrestins, β -arrestin1 and/or β -arrestin2, recruitment to the receptor (Evron et al., 2012). β -arrestin binding of the receptor results in GPCR internalization into clathrin-coated pits and then vesicles in a process known as endocytosis (Hanyaloglu & von Zastrow, 2008; Vilardaga et al., 2010). Endocytosis is a primary functional consequence of GPCR activation in which down-regulation of the amount of GPCR localized to the plasma membrane occurs in order to desensitize signal transduction via uncoupling from G proteins (Shenoy et al., 2007). This internalization of activated GPCRs is resolved by either recycling back to the plasma membrane or degradation (Kendall & Luttrell, 2009).

Many GPCRs can also be categorized into one of two classes based on ligand-induced GPCR interaction with β -arrestins. Class A GPCRs, such as the β_2 -adrenergic receptor (β_2 -AR), possess higher affinity for β -arrestin2 compared to β -arrestin1 and interact with both β -arrestins in a transient manner (Luttrell & Lefkowitz, 2002; Shenoy et al., 2007). Although β -arrestin diffuses to clathrin-coated pits with the receptor, the β -arrestin then rapidly dissociates from Class A GPCRs while the receptor persists as endosomes within the cytosol (Luttrell & Lefkowitz, 2002; Shenoy et al., 2007). Class B GPCRs, such as parathyroid hormone receptor type 1 (PTH₁R), exhibit strong and equivalent affinity for both β -arrestin1 and 2 (Luttrell & Lefkowitz, 2002). Dynamic divergence of GPCR classes exists via the fact β -arrestins internalize with the Class B receptors into endosomes unlike their Class A counterparts (Vilardaga et al., 2010). Differences in β -arrestin interactions with Class A and Class B mediate receptor fate (Kendall & Luttrell, 2009). These trafficking paradigms influence signaling events and underscore the major role of β -arrestins as scaffolding proteins in signal transduction.

Differential trafficking patterns of GPCR classes have been described for many receptors and the β -arrestins that dictate receptor sequestration (Ferguson & Caron, 2004; Hanyaloglu & von Zastrow, 2008; Kendall & Luttrell, 2009; Magalhaes et al., 2011). Real-time quantification of β -arrestin recruitment to the plasma membrane, colocalization with the receptor, as well as receptor internalization magnitude and rate, have each been examined using multiple approaches, yet have not been examined simultaneously within one assay platform. Herein, we describe the first method capable of quantitating all of the above phenomena as well as additional metrics in live-cell and real-time β -arrestin mediated internalization of Class A and Class B receptors. Reliable separation of Class A and B receptor types using our quantitative approach strengthens the stringency and reliability of this method. Although our method currently lacks high-throughput capacity, the high content analysis delivers multiple data features, including receptor internalization rate, β -arrestin colocalization duration, localization and aggregation of endosomes, and cellular movement. As a proof of concept, we tested our analytical method against classical Class A (β_2 -AR) and Class B (PTH₁R) GPCRs following activation with their canonical ligands, isoproterenol and hPTH₁₋₃₄, respectively. We performed this analysis in cells at multiple concentrations to examine the concentration dependency of internalization kinetics and also combined agonist treatment with antagonists specific to each receptor in question to confirm the specificity of the internalization kinetics.

Methods

Transient Transfections of HEK293 Cells

HEK293 cells (a gift from Dr. Anna-Liisa Nieminen) were maintained for up to 20 passages on 10 cm culture dishes in DMEM growth medium supplemented with 10% fetal bovine serum and gentamicin. Cells were maintained at 37°C in a 5% CO₂ atmosphere and were passaged by trypsinization every 3–4 days. HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to manufacturer's recommendations with 2 µg of either human β_2 -AR-YFP (kindly provided by Michel Bouvier) or human PTH_{1R}-YFP. Both experiments used 1.5 µg β -arrestin2-dsRed (Luttrell et al., 2001).

Preparation of Assay Plates

Prior to cell plating, sterile Costar black walled clear bottom 96-well plates (Corning #3603) were collagen coated by adding 50 µL collagen/acetic acid mixture to cover the bottom of each well. Collagen solution contains 800 µL of rat tail collagen (Becton, Dickson and Company, Franklin Lakes, NJ, USA) in 49.2 mL of sterile 2% acetic acid in distilled H₂O, sterilized using a 0.2 µm filter sterilization unit. Plates were incubated at room temperature for 1 h, after which the collagen solution was aspirated, and each well was washed twice with 100 µL of 1× HBSS. The transfected cells in 10 cm plates were trypsinized and seeded into collagen coated 96-well black walled clear bottom plates in growth medium at a density of 1×10^3 cells/well. This low density was necessary to properly image cells for computational quantitation as we wanted to isolate individual cells and to prevent the formation of a monolayer or large multicellular complexes. This low density was particularly important for visualizing agonist-induced changes in cellular morphology.

Live Cell Time Resolved Microscopy

Twenty-four to 48 h following seeding the assay plate, InCell microscopy was executed using an InCell 2000 Analyzer High Content imaging system (GE Healthcare, Livonia, MI, USA) with a QUAD2 polychroic and environmental controls (CO₂ + plate heating). Cells were stained with 250 nM of Hoechst 33342 in HBSS (Anaspec, Inc., Fremont, CA, USA) 5 min prior to agonist addition. Media was aspirated from the wells to be assayed and replaced with 100 µL of HBSS. Once optimal fields were selected based on cell density and relative dual fluorescent expression, 100 µL of solution containing ligand at 2× concentration [isoproterenol (Tocris Bioscience, Bristol, UK), hPTH_{1–34} (Bachem, Inc., Torrance, CA, USA), or hPTH_{7–34} (Bachem, Inc.)] was added at time point zero rendering a final ligand concentration of 1 µM.

To demonstrate inhibition of receptor internalization, antagonists were preincubated for 1 h prior to submaximal concentrations of either ligand (10 nM for isoproterenol and hPTH_{1–34}) as described above. For β_2 -AR, 100 µL of 2× concentration of ICI-118,551 (Sigma-Aldrich, St. Louis, MO, USA) was added 1 h prior to render a final concentration of 1 µM. Following the 1 h incubation, 200 µL of 2× isoproterenol was added to result in a final concentration of 10 nM followed by cell imaging. For PTH_{1R}, 1 µL of hPTH_{7–34} (Bachem, Inc.) was added

at a 100 $\times$ concentration to 99 μ L of 1 $\times$ HBSS 1 h prior to stimulation (final hPTH₇₋₃₄ concentration is 10 μ M) with 100 μ L of 2 $\times$ hPTH₁₋₃₄ for a final concentration of 10 nM.

Images were acquired using a Coolsnap K4 camera with 2 $\times$ 2 binning (output image 1024 $\times$ 1024 pixels, 0.370 μ m per pixel using a 40 $\times$ Nikon 0.6 NA Extra Long Working Distance objective). The sampling z height (depth of field) of this objective is 1.55 μ m. The YFP, dsRed, and DAPI filtersets were used to capture receptor, β -arrestin2, and nuclei images respectively into channels 1, 2, and 3 every 30 s (Figs. 1, 2). The fluorescence exposure was determined based on a maximal signal above 1,000 relative fluorescent units (which represents 25% of the dynamic range of the 12-bit camera) for each channel and ranged between 0.5–2 s for each wavelength across multiple transfections. The total time required to acquire each frame includes exposure time as well as filter changes and ranged from 5–7.5 s per frame depending on exposure times. This is important to note for colocalization as increasing the amount of time between acquiring each wavelength (although only 0.5–2 s in our example) will decrease the resolution of any colocalization measurements due to the constant motion of internalized receptor complexes.

Multiparametric Object Segmentation

Image quantitation was performed using GE InCell Investigator 1.6 running on 64-bit Windows 7 (DELL Precision T3500 with an Intel XEON W3505 @ 3.2 GHz and 18GB RAM). To identify receptor and β -arrestin2 objects, respectively, the YFP and dsRed images were segmented using the granular (object) segmentation algorithm available in InCell Developer Toolbox 1.9 with a kernel size of 3 and sensitivity of 50. The receptor and β -arrestin2 images were output into separate channels (6 and 7, respectively). Colocalization events between the two were identified by a preprocessing macro (combine-min operator using input channels 6 and 7, output channel 8), followed by an intensity based segmentation on the binary result image (minimum threshold 1, maximum threshold 4,095). See Figure 2 panels A4–A6 and B4–B6 for an example of segmentation. This method for detecting objects is performed on the original data and is dependent upon local, not global intensity variations, thereby enhancing the ability to track cells that redistribute intensity in their cytoplasm over time. Thus, an increase in detection is correlated to an increase in granularity (which occurs upon internalization), not an increase in overall integrated intensity nor increased yellow fluorescent protein (YFP)-receptor transcription/translation. This definition is dependent upon the kernel size used and is the reason we chose three pixels (1.11 μ m). This method is similar to the grain identification used previously but now with increased temporal and spatial resolution (Haasen et al., 2006).

Cell Detection

After all objects of interest were segmented, the cellular region was defined. The entire cytoplasmic region (named ComboCellBody) was defined using a multistep algorithm to ensure reproducibility among experimental replicates and over time within each experiment. We needed to control for variance between transfection efficiencies and fluorescence intensity values, which affect intensity uniformly within an entire experiment. The phenomenon of receptor internalization transforms an initially diffuse cytoplasmic distribution into a very granular area as more receptors and β -arrestin2 objects internalize

over time within an experiment. For a detailed description of the methods used to define (segment) the cell body, the reader is referred to the Supplementary Methods.

Target Linking (Assignment of Objects to Cells)

To assign the receptor, β -arrestin2, or colocalized objects to cells, a one or two-level hierarchical target linking strategy was used. This strategy was necessary to assure that cells (and by extension, the objects identified within them) would remain linked to the cell. In this way, every measure was reported on a per-cell basis so that measures could be correlated with one another to answer basic questions about time-resolved receptor physiology. To see a tabular representation of this hierarchy, the reader is referred to Table S1 in the Supplementary Methods.

Cell Tracking

To keep track of individual cells over time, we used the cell tracking algorithm built in to Developer Toolbox. This algorithm results in the assignment of a unique LinkedTrackID to each cell detected that remains consistent across time. The simplest of these algorithms (and the one we used) is the proximity algorithm, which determines that a cell is the same based on its position from one frame to the next. See p. 5 of the Supplementary Methods for parameters used in this algorithm.

Data Output

The final data output is an array with all pertinent measures assigned to ComboCellBody. All measurements were assigned on a per cell basis. The final data output for this protocol was limited to the selection of ComboCellBody objects only and exported into Microsoft Excel (.xls) format. (See Supplementary Table S1 in Supplementary Methods for a summary of the measures.)

Data Analysis

Data exported from InCell Investigator Developer Toolbox were formatted in Microsoft Excel 2010 for use within Spotfire (Tibco Software, Inc., Palo Alto, CA, USA). The top row (containing redundant channel labels) was deleted, and three columns inserted after the Section column. These columns contained formulae for calculating Well, Field, and Time from the Section column generated by Developer Toolbox. For a detailed description of these text-based extraction formulae, the reader is referred to the Supplementary Methods. The formatted spreadsheets for each experiment were then compiled into one master spreadsheet for each treatment group. These master spreadsheets were then imported into Spotfire for analysis. Initial analysis consisted of quality control to remove poor quality (e.g., dead, mitotic, neoplastic) and untransfected/unresponsive cells (see a stepwise summary of quality control in Fig. 3). The first quality control removed cells that had very low or no expression for either YFP or dsRed using object count of YFP (receptor), then dsRed (arrestin) (depicted as the transition from Figs. 3A–3D). These filters were correlated with intensity-based measurements, but intensity as a measure was only used for the inclusion or exclusion of cells. This was necessary due to differences in transfection efficiency and complex phenomena such as variable imaging due to flat versus round cells and object

mixing over time. Since we are not using fluorescence intensity as a measure but as a way to identify objects, the algorithm is relatively insensitive to photobleaching. Indeed, YFP intensity measurements were remarkably consistent through time, indicating we did not observe significant photobleaching (data not shown). The second level of quality control was to remove incomplete time series as evidenced by incomplete data for any individual LinkedTrackID (depicted as the removal of the yellow and blue traces in Figs. 3G–3J). Finally, we removed outlier cells that represented nonresponsive cells or cells with extreme values as determined by zero object count or cells with initial counts greater than ~200 objects, which was also an indication that the cell was either not transfected, the transfection efficiency was so low that it was undetectable, or the cell was neoplastic (e.g., multinucleated and “fried egg”). Neoplastic cells tend to be very large and are assumed to be abnormal. Similarly, mitotic and dead cells were also easily excluded as their spherical shapes resulted in low object counts, likely because the majority of these cells resided outside of the 1.5 μm imaging plane and were thus not imaged (data not shown). Figure 3 shows a representative image where only one cell was retained, which was chosen for visual clarity. Our actual ranges for inclusion varied and ranged from 19% to 71% with an average inclusion of 42% (Table 1). From a simplistic point of view, the fewer the cells and the higher quality of cells within a field, the more data that was retained. We determined through trial and error it was more robust to remove poor quality cells at the level of supervised analysis than via hard-coded thresholds during unsupervised detection—this was highlighted by an increase in the number of complete time points per LinkedTrackID upon moving quality control measures post-hoc, during Spotfire analysis (data not shown). Once per cell quality control was complete, average cell responses were calculated as the average among individual cells $\pm$ SEM.

Statistical Analysis

Statistics were performed within GraphPad Prism 6 (Graph-Pad Software, Inc., La Jolla, CA, USA). Linear regression (within the advanced nonlinear regression options) was chosen to evaluate the relationship between continuous variables (object count and time). For each receptor class, we computed the initial slopes of object formation over time (0–4.5 min) and tested them using the null hypothesis that β -arrestin2 and colocalized object formation rates are equal to the same-class receptor object formation rates. Our second linear regression analysis utilized time points 5–16.5 min and compared the slopes of each individual object counts over time to the value 0 (to test the null hypothesis that there is no net decrease in object count over time). Correlation was used to determine the degree of linearity between area and count for receptor, β -arrestin2, and colocalizations.

Results

In every time point, we first identified the objects formed by GPCR-YFP and β -arrestin2-dsRed. Note that diffuse fluorescence was not quantitated (a feature of the object/granular segmentation algorithm) as we used a small kernel size (minimum of 3 pixels), which biases detection toward local variations in fluorescence intensity. Following identification of GPCR-YFP and β -arrestin2-dsRed objects, we combined these images to identify the exact points of colocalization. All object measurements were taken from these data. Each object

was assigned to a cell using target linking so that each set of GPCR-YFP, β -arrestin2-dsRed, and colocalized objects were assigned to an individual cell. By binning objects to the cell that contained them, we were able to analyze measures on a cell-by-cell basis and facilitate quality control, while enabling the quantification of heterogeneity among cells. Each cell was identified by similar methods of detection using the GPCR-YFP signal (based on larger local variations in image intensity). This process was then done for every time frame and tracking of cells accomplished via assigning a unique LinkedTrackID that remained consistent over time. Our microscopy setup provided three fluorescent channels in three fields every 30 s for 30 min, providing 59 sets (or 177 frames) of quantitative data per field.

Real-Time Trafficking of Class A versus Class B GPCRs

We began by measuring the β_2 -AR and β -arrestin2 fluorescent endosome formation in response to the agonist isoproterenol and PTH₁R and β -arrestin2 endosome formation in response to hPTH₁₋₃₄ in transfected HEK293 cells using standard 40 $\times$ wide-field fluorescence microscopy. As seen in several previous studies (Luttrell et al., 2001; Luttrell & Lefkowitz, 2002; Ferguson & Caron, 2004; Perroy et al., 2004; Hudson et al., 2006; Shenoy et al., 2007; Ross et al., 2008), both isoproterenol and hPTH₁₋₃₄-treated cells are able to internalize receptor after 30 min exposure to agonist (Fig. 1). At later time points, β_2 -AR was not highly colocalized with β -arrestin2, whereas PTH₁R was extensively colocalized with β -arrestin2. Cells that received vehicle showed no obvious changes (Fig. 4). Figure 1 captures what is typical in many published studies where a representative cell is presented with isolated time points; yet, what happens at intermediate time points is often missed. Figure 2(A3/B3) (and Figs. 5, 6) depict the same cells after 3 min of agonist. It is immediately apparent that the cells are not at an intermediate between time 0 and 30, but a unique state is observed. Objects are small and localized near the plasma membrane. Considering how much detail was missing from Figure 1 in terms of the total biological phenomenon, and to provide a mechanism to present replicates and error, we designed a method to fully quantitate the time resolved microscopy data.

Our goals for the software were to simply provide an analysis method that reports localization and colocalization of proteins, basic information on the morphology of these foci, and to reduce investigator bias when presenting results. This was achieved by three basic computational algorithms combined with manual analysis of the quantitative results.

Granular (Grain-Based) Segmentation

Each image (one per filter set per time point) acquired by the microscope is a 12-bit (4,096 levels) grayscale bitmap. Our first step is to identify objects within the bitmap that exceed the noise threshold using a granular segmentation algorithm. This algorithm detects fluctuation in the local pixel neighborhood of every single pixel and identifies small, bright objects. The output of the segmentation processing is a 1 bit image (black and white) where every object is defined as white on a black background (Figs. 2, 7). Intensity data are not discarded but instead are available for further analysis, using the black and white segmented image as a mask to define where to measure intensity in the original image. However, we did not incorporate intensity in our quantitative analysis, as intensity is prone to experimental artifacts. This approach was done for every wavelength (3 per field), and every

time point (59 per field, every 30 s for 30 min). Grain-based segmentation is a powerful technique to identify objects within cells, and it has been used successfully in automated image analysis (Haasen et al., 2006; Ross et al., 2008). A second segmentation identified the cell, using an alternate method from object detection (see Supplementary Methods, pp. 1–4). Similar methods to define cellular regions have been successfully accomplished by others using Gaussian filtering and region growing to precisely map the outline of cells from fluorescent images to produce cell counts, cell location, and cell border measurements (such as area and form factor) (Fenistein et al., 2008). After all four segmentations (cell body, nuclei, receptors, and β -arrestin2) were complete, the receptor and β -arrestin objects were assigned to cells using target linking (see Supplementary Methods, p. 4, and Table S1).

Subtractive Masks to Define Colocalization

Subtractive masks have been used successfully by others to determine colocalization (Kwon et al., 2012). By using a subtractive mask (Combine-min point operator in Developer Toolbox) between the segmentation results for receptor and β -arrestin2 channels, we are able to determine areas where these two proteins coexist. This subtractive mask is equivalent to the Boolean logical “AND” statement, but for an entire image comparison. These colocalization events were treated as a fifth object in segmentation, target linked to cells, and included in the analysis. As we are measuring the colocalization of objects defined from two separate wavelengths, there is the possibility of misregistration (shift in position) between the wavelengths due to chromatic aberration of our optical system. While we were able to correct for this in the z dimension consistently using a defined offset for each individual wavelength during acquisition, we were unable to correct for lateral misregistration (in the x and y dimensions) using the currently available platform. Nonetheless, the use of temporal tracking greatly increases the usefulness of colocalization in wavelength/chromatic aberration-uncorrected images as changes over time provide information on relative changes in colocalization, despite any errors in lateral misregistration. Future studies could correct any very small misregistration by vector-based calibration of the x and y dimensions using fluorescent beads (Dorval et al., 2010).

Computational Calculation of Measures

Data are derived comprehensively in a per cell fashion, summing individual receptor, β -arrestin2, and colocalization data per cell (Figs. 5, 6, 8–13). As measures were performed on a per-cell basis, we are able to correlate diverse measures with one another (e.g., receptor count with β -arrestin2 count, colocalization count with cell size, etc.). As an example of this multiparametric analysis, see Figure 14, where changes in cell shape over time are demonstrated using the measures Cell Area and Form Factor [equation (1)].

$$Form\ Factor = \frac{4\pi[Area]}{[Perimeter]^2}. \quad (1)$$

Form factor is a measure of complexity, ranging from 0 to 1. Increasing values indicate simpler shapes, with a circle having a value of 1.0.

Supervised Analysis

The result of the above steps of the algorithm is a large matrix (spreadsheet) of measures reported for every single cell and time point analyzed. This large amount of data underwent manual analysis coupled with methodical quality control. Our quality control exclusion criteria were quite simple. There must be (1) a positive receptor object count (which also verifies the presence of the receptor transgene), (2) the cell must contain the β -arrestin transgene, and (3) no missing data (i.e., broken traces) for receptor count throughout time (Fig. 3, Table 1). There was a moderate amount of data excluded (average of 42% of all detected cells were included), but we always retained at least one fully analyzable cell per field. This error rate corresponded with manual survey of 5–6 fields per dataset (Fig. 4). Following quality control, we determined the appropriate measures to report based on relevance to the research question of GPCR/ β -arrestin2 interaction physiology.

β -arrestin2 Objects Discriminate Class A from Class B GPCRs

In the final groups of cells selected for analysis of each receptor and treatment, we quantified the receptor, β -arrestin2, and colocalization count kinetics along with other cellular morphometrics. At the saturating concentration of 1 μ M isoproterenol for the β_2 -AR receptor, receptor count kinetics showed a rapid increase, plateauing at 4 min followed by 14 min of equilibrium and a third phase of mild decrease indicative of endosome coalescence (Fig. 8A). Unlike receptor object count, β -arrestin2 count kinetics displays a peak of endosome detection at roughly 2 min following isoproterenol stimulation (Fig. 8B). This was followed by a rapid decrease in β -arrestin2 object number of objects for 14 min, which is indicative of disengagement with the receptor and adaptation back to a diffuse cellular distribution (Fig. 8B). Finally, β -arrestin2 object formation equilibrium was achieved after 16 min. The colocalization of β -arrestin2 and β_2 -AR follow a similar trend to β -arrestin2 counts through time, but kinetic trends (speed, maxima/minima location) were intermediate to receptor and β -arrestin2 and reached a peak at 3 min (Fig. 8C). By overlaying all objects identified in the 1 μ M isoproterenol group together, it becomes apparent that colocalizations form at a count that is approximately 50% of the total arrestin count and 33% of the receptor count (Fig. 8D). Although some β -arrestin2 objects are absent prior to the time of recruitment and may or may not persist to a diminished degree for the 30 min window observed, from the dataset it is clear β -arrestin2 recruitment, colocalization, and release of the Class A receptor are narrow windows that are easily missed in microscopy of fixed cells at a limited number of time points.

PTH₁R receptor internalization in response to saturated ligand (1 μ M hPTH₁₋₃₄) follows a similar increase in object formation reaching maximal object detection at 5 min, a minute later than the β_2 -AR (Fig. 8E). Compared to β_2 -AR, aggregation of PTH₁R receptor object persisted, resulting in only a mild decrease in the total receptor object count for the rest of the experiment. β -arrestin2 object formation peaks at roughly 4 min followed by a slight decrease in counts. β -arrestin2 counts persist at a greater number than β_2 -AR for 30 min and follow a similar trend to receptor count (Fig. 8F). Colocalizations between PTH₁R receptor objects and β -arrestin2 objects increased to reach a maximum at 7 min, as compared to the 3 min in the β_2 -AR group. By overlaying all objects detected in the group of cells treated with 1 μ M hPTH₁₋₃₄, it becomes apparent that a much larger proportion of receptor and β -

arrestin2 objects overall are colocalized in comparison with the β_2 -AR group (Fig. 8D, 8H). Colocalizations comprised approximately 75% of receptor objects and a varying number (66–90%) of β -arrestin2 objects throughout time (Fig. 8H). Please see Figure 10 to observe individual cellular kinetic responses of count versus time.

To confirm the specificity of the response to the Class A or Class B receptor of interest, we extended our analysis to include a lower concentration of ligand as well as the inclusion of an antagonist for each receptor class (Figs. 8A–C, 8E–G). It was observed that lower concentrations (10 nM) of each receptor agonist produced both a decrease in the initial rate as well as a delay in the maximum number of receptor puncta on average per cell in comparison to the 1 μ M concentration. This less than maximal response was completely abrogated by concomitant pretreatment of selective competitive antagonists for Class A or Class B, ICI-118,551 1 μ M or hPTH_{7–34} 10 μ M, respectively. Receptor internalization inhibition within our system was only possible at submaximal stimulation with our reference ligands. Despite efforts to elicit partial receptor internalization in our system, we found this internalization phenomenon seems to occur at a specific threshold whereby receptor internalization occurs or is blocked.

In general, peak counts were greater for Class A compared to Class B for both receptor and β -arrestin2, but similar for colocalizations (Fig. 8I–8K). Peak number of receptor objects was reached at 5 min for Class A and 5 min for Class B. After reaching a peak, β_2 -AR counts decreased over time; this is compared to a sustained receptor object count for PTH₁R. Persistent colocalization of β -arrestin2 and PTH₁R further validates the classical perspective of Class B GPCR internalization. Interestingly, β -arrestin2 in both classes displayed similar responses (Fig. 8J), suggesting that the net formation and degradation of β -arrestin2 foci (objects) is not solely driven by β -arrestin2 interaction with the receptor. Nonetheless, this finding agrees with existing data that demonstrate prolonged interaction of β -arrestin with Class B GPCRs as our colocalization counts do recapitulate the phenomenon of persistent colocalization in this group compared to Class A GPCRs (Fig. 8K). In summary, comparing the results with published Class A and B GPCR experiments, expected similarities and differences between Class A and B were identified but with a very high level of kinetic resolution and in a defined temporal approach (Luttrell & Lefkowitz, 2002; Hanyaloglu & von Zastrow, 2008; Kendall & Luttrell, 2009; Magalhaes et al., 2011).

We achieved a temporal resolution of 30 s and a spatial resolution of 1.11 μ m (Nyquist sampling limit of 3 pixels per object). There are likely some minor artifacts due to the time dilation between sampling each wavelength (~500 ms to 3 s depending on exposure time). Therefore, differences in timing greater than 1 min (or two time points) should be considered as significant. Based on average counts of objects detected in stimulated and nonstimulated cells (Figs. 8 and 11, respectively), we estimate our signal to noise ratio for object count at approximately 7:1 (135 peak counts/20 peak count error).

As the velocity of receptor internalization (V_i) is one potential metric of agonist efficacy, we also analyzed the initial rate of change of object counts (internalization) for Class A and B. As displayed in Figure 9, we calculated the slopes for the initial 4–7 time points (averaged over 5–6 cells for each receptor type) via linear regression, plotting the calculated

lines as solid lines in Figures 9A and 9D. When each class's initial rate of β -arrestin2 or colocalized object formation is compared to the rate of receptor object formation, it is apparent that there is GPCR class divergence. For our Class A prototype receptor β_2 -AR, there is a significant difference between receptor internalization rate and the formation of colocalized objects (Fig. 9B). This observation is thought to be caused by receptor objects dissociating rapidly with β -arrestin2 upon endocytosis of the β_2 -AR. In the Class B dataset, there is no such disconnect between receptor internalization and formation of colocalized foci, indicating that Class B GPCR internalization is tightly linked with its colocalization with β -arrestin2 (Fig. 9E). There is, however, a significant increase in β -arrestin2 object detection when compared to the referent PTH₁R-YFP. This rate is similar to the β -arrestin2 rate in the β_2 -AR dataset, which suggests congruence between the initial rates of β -arrestin2 recruitment in both classes. Therefore, our method and data quantify receptor internalization kinetics and at the same time can be used for quality control measures to demonstrate the consistency of β -arrestin2 kinetics between divergent receptor classes when stimulated with their respective reference ligand.

We next sought to quantify the differences in kinetics of receptor foci formation and degradation following the maximum observed counts. Similar to the calculation of initial internalization velocities, we performed partial linear regression for receptor, β -arrestin2, and colocalized object counts from 5–16.5 min. This interval was chosen as (1) it represents the maximal rate of decrease following maximum object formation and (2) this interval was linear for all datasets. The dotted lines in Figures 9A and 9D represent the calculated regression equation for this interval, and the slopes are plotted below in Figures 9C and 9F. As expected, we observed a significant decrease in all post-maximal object formation rates (V_{pm}), with the notable exception of PTH₁R receptor objects, whose rate did not significantly differ from zero. This finding supports previous work demonstrating that Class B receptor endosomes are persistent following internalization (Kendall & Luttrell, 2009).

To control for potential hyper-endogenous receptor internalization in the absence of exogenous ligand stimulation—a concern in a system of overexpression of β -arrestin2—nonstimulated controls for five cells from three separate transfections for β_2 -AR and nine cells from two separate transfections for PTH₁R were quantitated in the same approach as stimulated cells (Fig. 11). Equal volume ligand vehicle (DMSO or H₂O for isoproterenol or hPTH_{1–34}, respectively) was added in the same manner as ligand application, and the imaging duration and quantification followed. No significant object formation was apparent after 30 min of vehicle addition, indicating the cellular responses were agonist specific (Fig. 11). These nonstimulated controls verify our quantification of objects as ligand-mediated endosome formation.

Relating Object Count to Total Area Occupied by Object

Measuring the number of objects per cell is useful, but taken alone it does not allow one to make any claims about the net flux of signaling, particularly with regard to endosome coalescence. For Class A, peak receptor area was achieved at 6 min, 2 min after peak count (Fig. 12). β -arrestin2 area peaked at 2 min, as did β -arrestin2 count (Figs. 8, 12). Again, colocalizations peaked at a time point between receptor and β -arrestin (2.5 min) and had an

equilibrium phase of ~10 min, which was followed by a decline. Between 14 and 18 min, we observed a curious transient decrease in object area that was not seen in the β -arrestin2 count plots.

For Class B, peak receptor area was achieved around 10 min, 4 min after peak count. β -arrestin2 area peaked at 6 min, 2 min after peak count, followed by a slow decline. Colocalization area in this case continued to increase for the first 10 min and remained stable for the remainder of the experiment, again demonstrating a sustained interaction between β -arrestin2 and receptor.

Individual Object Sizes Discriminate Class A from Class B GPCRs

To further bolster our analysis, especially with regard to endosome coalescence, we looked at the response of individual object size to agonist stimulation over time. Previous work was able to bin object size in terms of the number of pixels per object, but here we show a continuous measure of size (Haasen et al., 2006). By dividing Area by Count, we can effectively calculate the mean object size on a per-cell basis to demonstrate the effect of dynamic receptor and β -arrestin2 trafficking flux on individual object size (Fig. 13). This mean individual object size is reflective of the progression of the signaling process (Hanyaloglu & von Zastrow, 2008; McCaffrey et al., 2009). Initially, receptor is diffuse at the plasma membrane before agonist and is resultantly detected along the cell borders due to sharp demarcation against background; subsequently, initial receptor objects on average occupy a larger area per object. However, per object size then decreases rapidly as receptors and β -arrestin2 then form pits at the plasma membrane followed by early endosomes, increasing count more quickly than area. These objects are small compared to late endosomes where aggregation of many individual endosomes has occurred, and there is a greater increase in area relative to count. Indeed, we observe after an initial 2 min period of decrease in size that there is a slower increase in size of receptor objects, indicating vesicle aggregation. Interestingly, receptor objects for both Class A and B GPCRs studied here show the same kinetics. However, only Class B GPCRs induce a similar kinetic profile in the β -arrestin2 objects and colocalization foci over time. Class A shows only minor changes in β -arrestin2 size over time, which extends into the colocalization average size as well. This is in support of the necessity for β -arrestin2/Class B GPCR interactions prior to internalization and is direct evidence demonstrating prolonged receptor/ β -arrestin2 interaction. It also demonstrates the phenomenon of endosome aggregation or coalescence, apparent in the original microscopic images of PTH₁R (Fig. 5).

GPCR Classes Diverge in Cellular Motility and Morphology in Response to Agonist

By observing the Cell Area and Form Factor measures (Fig. 14), we were able to quantify an observed receptor-specific induction of cellular rounding. β_2 -AR transfected cells underwent only a small increase in Form Factor, indicating that cell shape simplified only trivially during the 30 min of image acquisition. However, PTH₁R when stimulated underwent a significant simplification of its shape (retraction of podia, general rounding), which is reflected by a concomitant decrease in area and increase in Form Factor. Analysis of cell movement is easily extracted as the x and y position of each cell is determined for every time point. Figure 15 shows the overall movement of cells from different experiments within the

field of imaging. Taken together, these data indicate that although both receptors communicate with the cytoskeleton, activation of the β_2 -AR leads to a minor increase in cell motility and marginal cell rounding, while activation of the PTH₁R leads to increased cell rounding without as much cell motility.

To understand the variance we observed within each dataset, we performed a multiparametric analysis of object count with cellular size (as defined by the ComboCellBody mask). The data were analyzed by linear regression to calculate correlation. In both Class A and B, receptor count correlated positively with cellular size at all time points ($R^2 = 0.510$, $p < 0.0001$ for β_2 -AR and $R^2 = 0.649$, $p < 0.0001$ for YFP-PTH₁R) (Fig. 16). Simply put, larger cells (presumably with higher total protein) produced more objects across all time points. This suggests that GPCR-YFP transfection efficiency may play less of a role in determining the magnitude of receptor internalization in response to ligand.

As expected, there is a divergence between the receptor subtypes with regard to the correlation of β -arrestin count and cellular size. In our prototypical Class A GPCR β_2 -AR, we observed no significant correlation between β -arrestin2 count and cell size, whereas there was a significant correlation between the two in the Class B prototype PTH₁R ($R^2 = 0.001$, $p = 0.564$ and $R^2 = 0.667$, $p < 0.0001$, respectively). We interpret these data as additional evidence in support of the prolonged interaction between β -arrestin2 and GPCR in Class B and the fleeting interaction in Class A (Fig. 16).

There are a very large number of metrics that can be gathered from the analysis. For simplicity and validation we focused on object count and began to explore object area and size as well as whole cell morphology. It is feasible to extend the analysis to subcellular compartments, e.g., by extending the nuclei mask to create a perinuclear compartment. Preliminary analyses indicated that retraction of the cell borders after agonist creates significant overlap between compartments. This situation could be resolved by analyzing very wide cells, customizing the compartment diameter across time to account for the contraction of cellular membranes in the x and y dimensions, or increasing magnification and resolution.

Discussion

Our analysis demonstrates there are major differences in the short time- and small size-scale events between Class A and B GPCRs. As opposed to current methods, this method can simultaneously quantitate protein trafficking, cell morphology, and movement as measures of receptor activation. Additionally, this method affords a high kinetic resolution and ability for automation. This live cell real-time analysis of receptor internalization following ligand mediated activation is applicable to address many questions in basic science research and inform drug discovery initiatives. This method provides real-time tracking of fluorescently labeled proteins upon stimulation to obtain internalization kinetics and can be extended to define the subcellular localization of proteins such as β -arrestins. The high kinetic resolution of this approach provides analytic flexibility that can eliminate the need for cell fixation. Because our system did not employ automated liquid handling, there is very little early time

event data in the set. Nonetheless, it is apparent we were able to capture the initial stages of signaling despite the lack of pre-injection data for each individual cell and potential for ~20 s delays in initial image acquisition.

This method focused on a coarse resolution approach to identify and study the limits and strengths of real time per cell quantitation. We now understand that there are very rapid and complex events that happen in the first 4 min that are not traceable with the current microscopic experimental setup (low 0.6 NA of the 40× objective limits pit detection and lack of liquid handling delays the start of our acquisition ~20–30 s). To improve the capture of these fleeting changes in a delicate system—to quantify early interactions—many improvements could be made, such as faster time series acquisitions, higher resolution optics, and/or confocal or deconvolution to improve z axis resolution, which could allow true four-dimensional analysis ($x + y + z + \text{time}$). This combinatorial approach would utilize all the microscopic advantages provided by automated imaging systems such as the InCell 2000. The same can be said for long-term events that happen over hours such as termination of prolonged second messenger signaling, receptor/ β -arrestin recycling rates, and degradation (Hanyaloglu & von Zastrow, 2008; Vilardaga et al., 2012). Perhaps, not unexpectedly, you cannot acquire all data on all scales at the same time.

Our measurement represents a very large increase in the number of samples that are used to estimate error. Where previous methods usually relied on several images, we are estimating error from multiple experiments, multiple cells, and multiple images. These types of data are especially well suited to coupling with other high-resolution techniques measuring ligand binding and second messenger fluxes (Ross et al., 2008; Dale & Ferguson, 2011). Most previous work used fixed cell end-point data with an apparent temporal resolution no better than 5 min. It should be noted that using fixation methods creates a much larger time dilation than the 2–4 s it typically takes to acquire a single live cell time point. Recently, time-resolved impedance assays have shown ligand bias effects though the β_2 -AR as an integrated measurement of changes in cellular morphology (Stallaert et al., 2012). It would be quite interesting to qualify and quantify the relationship of impedance changes to specific morphological changes as observed directly using these two methods in tandem. This technique is also well suited to study the impact of G protein and G protein regulators' ability to regulate signalosome trafficking (Peterson et al., 2000; Ghosh et al., 2003).

Strength of signal effects elicited by different ligand concentrations may be determined by quantitating the changes in magnitude and rate of β -arrestin recruitment to the plasma membrane and duration of colocalization as well as receptor internalization velocity (Barker & Benovic, 2011). Phenomena such as sustained PTH_1R -mediated cAMP generation following hPTH_{1-34} administration could be correlated with persistence of $\text{PTH}_1\text{R}/\beta$ -arrestin endosomes following wash out (Ferrandon et al., 2009; Vilardaga et al., 2012). This increase in cAMP could then be analyzed in context with components of the endosomes providing the machinery for prolonged signaling such as adenylyl cyclases and G proteins themselves. This example demonstrates quantifiable divergence in β -arrestin mediated GPCR internalization between GPCR class types and will allow us to answer questions regarding desensitization (Claing & Laporte, 2005).

As we have demonstrated the sensitivity of this method for detecting canonical β -arrestin-receptor interactions via monitoring colocalization kinetics in one prototype receptor from Class A and one from Class B using canonical agonist ligands, it will be interesting to design or discover ligands that increase β -arrestin objects persistent for Class A GPCRs or ligands that prevent persistent β -arrestin objects while preserving internalization for Class B GPCRs. We expect that many aspects of ligand bias could be revealed by careful quantitative temporal and spatial analysis of the immediate early regulators of GPCR signaling (Tesmer, 2010). For instance, what effects do ligands of different chemical classes that produce different phenotypes have on receptor and β -arrestin trafficking kinetics? This question's importance is highlighted by the observation that formoterol increases mitochondrial function while many other selective β_2 -AR agonists do not elicit the same effect (Wills et al., 2012).

This method allows for a robust survey of signaling and trafficking processes and especially kinetics with a large number of applications. Measurements of receptor internalization kinetics may be correlated with activation of downstream targets. For example, time course Western blot analysis of Erk activation by hPTH₁₋₃₄ has demonstrated that the maximal phosphorylation event coincides with maximal PTH₁R/ β -arrestin colocalization at $t = 10$ min (Tohgo et al., 2003). With this method, the reverse approach can be applied such that detection of protein activation may be expected at time points acquired based on receptor internalization and β -arrestin distribution.

In our experimental design, previously internalized receptor objects (those already present and attributed to nonligand-induced internalization) represent a background signal. It is thus our belief that monitoring the initial slope of internalization is a more useful measurement than is measurement of the absolute internalization count at time = 0. However, we caution those who wish to extend this method into their own system that they consider carefully any normalization scheme between divergent cells. We have avoided normalization in our data analyzed for kinetics as we aim to describe the large amount of variation inherent between cells that have been transiently transfected. Indeed, we suggest a large benefit to studying ligand-receptor interactions as we have by including multiple concentrations (concentration response) and agonist-antagonist competition as experimental controls in addition to the standard vehicle control to determine the kinetics of their receptor system.

This high-content methodology is not necessarily a high-throughput methodology due to the necessity for high-quality images at short time intervals. Computational analysis can take half an hour per image stack on a modern workstation, even before data analysis can begin. Subsequent development and parallel processing could increase this speed, along with application of unsupervised exclusion of low quality cells in analysis; however, the latter would come at the cost of accuracy and would necessitate extended statistical validation. Additionally, a stable expression system would enhance the number of quantifiable cells due to enhanced consistency in genetic expression compared to our transient system. Increasing the cellular density on the plate could also increase the efficiency of this method; however, for the information gained in numbers of cells analyzed, the investigator should be cognizant of cell-cell interactions' potential to alter the cellular morphological and receptor/ β -arrestin internalization response. This modification would be crucial in beginning to understand the

role of cell-cell communication in GPCR signaling (Kaya et al., 2012). High cell densities are also more difficult for the software cell tracking system. However, the major problem in cell tracking is collision events, and performing experiments on a stable, stationary monolayer could bypass this limitation.

The lack of confocal images makes identifying very fine detailed objects difficult. Using a high content confocal imager, Hudson et al. (2006) have been able to resolve pit formation from endosome internalization. Of course, this increase in spatial resolution came at the cost of temporal resolution, necessitating fixation before image acquisition. The use of modest numerical aperture (NA) and moderate magnification allows for persistent identification of objects. We have avoided the problem inherent in high NA, small depth-of-field confocal experiments where objects can move out of focus by trafficking through the z plane. Future studies could combine the benefits of increased lateral resolution and sensitivity afforded by high NA objectives by computationally combining z stack images (maximum intensity projection, etc.). It is also important to note that the current technique is significantly more likely to detect and quantify endosomes versus clathrin-coated pits. This fact is due to our limit of resolution ($0.37\text{ }\mu\text{m}$ per pixel results in Nyquist limit of resolution of $1.11\text{ }\mu\text{m}$) and to the smaller, dimmer nature of these pits in comparison to endosomes (Hudson et al., 2006).

This method can be applied to a myriad of signaling questions and is especially well suited to GPCR signaling (Bockaert et al., 2010; Magalhaes et al., 2011). For instance, the coupling of GPCRs to regulators such as G proteins, β -arrestins, additional scaffolding proteins, and enzymes can all be assessed using fluorescently-tagged versions of each in combination. The further we advance our understanding about β -arrestins, the more it has become apparent that these regulators of receptor internalization and eventual desensitization also bestow unique signaling properties to GPCRs. Although only relatively coarse spatial distribution ($1.11\text{ }\mu\text{m}$ was our Nyquist limit, $0.601\text{ }\mu\text{m}$ Rayleigh) can be detected, far from the precise spatial distribution capable in confocal microscopy or super-resolution techniques, the extensive automated quantitation and high kinetic resolution of this method performed on live cells is still very descriptive of the overall kinetics. Although exact or relative subcellular spatial distribution of protein is lacking in this assay platform, protein redistribution following stimulation is detectable.

Colocalization events can be difficult to positively identify in wide-field microscopy as the larger depth of field in comparison to confocal imaging means an object that appears in the same x and y position may actually be present in a different z position. Nonetheless, the persistence of a colocalized object over multiple time points supports the argument that the individual fluorophores comprising it are truly colocalized (within the limits of lateral resolution) and enables the ability to measure relative changes in magnitude of true colocalization. Spuriously detected colocalizations (those formed by fluorophores at different planes) should appear and dissipate in a Gaussian distribution and thus would affect the magnitude of detected object count equally at all times of analysis. Therefore, spurious detections would only affect the intercept of kinetic analysis, not the slope, and we argue the slope is a more useful measurement of colocalized β -arrestin/receptor internalization kinetics.

One of the major problems in turning an endpoint or kinetic assay into a high content informatics assay is metric expansion. In this article we attempted to limit this expansion by looking at only two protein pairs and a limited number of metrics/measures (count, total area, and average area for objects—location and area for cells). However, the total number of metrics is limited only by the vision of the investigator and available mathematical operations. There are a large number of measures for any given object and when you consider a combinatorial approach to analyze measures, the possible comparisons increase multiplicatively, not additively. We hope and expect that the biology will drive the kind of metrics that are most descriptive, and we recognize that we have only scratched the surface of measures related to object characterization in GPCR function.

We purposefully did not use fluorescent intensity as a metric due to the large number of factors that can influence intensity (bleaching, protein degradation, quenching, focusing). However, we can still monitor intensity for quality control purposes. Indeed, we observed stable object fluorescent intensity though the 30 min time course (data not shown). This was expected as the fluorescent protein constructs used are relatively resistant to photobleaching, and we balanced the exposure time to minimize the total exposure of the cells to the light source while providing adequate signal-to-noise ratios for quantitative analysis.

Although we are only tracking two discrete proteins, the kinetics we measured involve the entire host of GPCR regulation and trafficking machinery. GPCR signaling is not a linear cascade. It is instead a kinetic process involving amplifiers, feedback loops, and dynamic interactions across the chemical to cellular scale and includes interactions between ligands, proteins, and intracellular organelles. Many of the endpoint assays used to study GPCR signaling observe steps that are too far downstream to be predictive of upstream events such as subtle changes in ligand structure or receptor cross-talk. We hope that this method will add another tool to analyze patterns of upstream signaling that can be used to better understand the integration/interaction of pathways and perhaps predict downstream functional biology.

Despite our assay platform limitations, this technique proves the ability to condense and analyze multiple replicates robustly. In our case, several different transfections on different days were compiled into the final dataset, providing a high degree of accuracy in error estimation. This approach is essential to determine the degree of consistency in a given population of cells within certain experimental systems. Identification of ligands that activate orphan GPCRs may be tracked in a real-time fashion, and subtle yet potentially significant difference can quickly be teased apart due to the real-time nature of the assay platform, enabling drug discovery efforts.

Conclusion

We describe a novel time-resolved fluorescent microscopic acquisition coupled with high content multiparametric object classification to successfully discriminate Class A and B GPCR internalization characteristics with regard to their interactions with β -arrestin2. The applicability of this platform is broad, as a wide array of experimental questions beyond

GPCR signaling may be assessed, and the method provides the availability to finally correlate real-time GPCR signaling kinetics with protein mobilization kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Standard Imaging of YFP-receptor and dsRed- β -arrestin2 objects and colocalization.
Receptor and β -arrestin channels increase in granularity (formation of objects) over time.
Color legend: YFP-GPCR in green, dsRed- β -arrestin in red, Hoechst 33342 in blue.

Figure 2.

Time-resolved object segmentation of real-time fluorescent protein trafficking. In panels A1–3 and B1–3, original microscopic images formatted for display (false colors in parentheses, channels merged in A3): YFP-tagged receptor (green), dsRed-tagged β -arrestin2 (red), Hoechst-stained nuclei (blue). In A4–6 and B4–6, segmentation results overlaid on original grayscale images. Nuclei are outlined in blue and cell borders in cyan. Green, detected receptor objects (A4, B4); red, detected β -arrestin2 objects (A5, B5); yellow, colocalization of receptor and β -arrestin2 (A6, B6).

Figure 3.

Quality control summary. Shown are object counts detected in a single field over time for YFP-PTH₁R stimulated with 1 μ M PTH₁₋₃₄. Raw data are shown in the leftmost column (A-C), and the rightmost column (J-L) the single cell remaining in this field after filtering efforts were complete. In stepwise fashion, we discarded non-YFP GPCR transfected cells (as determined by low YFP intensity and/or low receptor count); shown in panels D-F. Next, non-dsRed β -Arrestin2 transfected cells were discarded (as determined by low dsRed intensity and/or low arrestin count); shown in panels G-I. Finally, traces coming from cells that had missing or incomplete data points over time were then filtered out, resulting in our final output of one cell from this particular well in panels J-L. This cell may be seen in context with other individual cells in Figure 10 (with identical color) and is included in the average rate for 1 μ M PTH₁₋₃₄-treated cells presented in Figures 4 and 5.

Figure 4.

Standard imaging of vehicle-treated controls. Receptor and β -arrestin2 channels show no increase in granularity (formation of objects) over time when no ligand is given. Color legend: YFP-GPCR in green, dsRed- β -arrestin2 in red, Hoechst 33342 in blue.

Figure 5.

β_2 -AR real-time imaging. Receptor and β -arrestin2 channels increase in granularity (formation of objects) over time. Color legend: YFP- β_2 -AR in green, dsRed- β -arrestin2 in red, Hoechst 33342 in blue. Segmentation of individual time points in final column is on the right.

Figure 6.

PTH₁R real-time imaging. Receptor and β -arrestin2 channels increase in granularity (formation of objects) over time. Color legend: YFP-PTH₁R in green, dsRed- β -arrestin2 in red, Hoechst 33342 in blue. Segmentation of individual time points is in the final column on the right.

Figure 7.

InCell Developer analysis summary. Screen capture of InCell Investigator Developer Toolbox Image View. This demonstrates the identification of all objects and masks used in the analysis. Channels 1–3 are the original microscopic images [YFP, dsRed, Hoechst (DAPI)]. Channel 4 is empty and is used for preview only. Channel 5 shows the output of the cellular segmentation (ComboCellBody). Channel 6 shows the output of YFP-receptor segmentation, false colored green. Channel 7 shows the output of dsRed- β -arrestin2 segmentation, false colored red. Channel 8 shows the output of the Combine-Min operator, used for subtractive colocalization detection, false colored yellow. Channel 9 is the result of the Information Equalization step applied to the YFP-receptor image, an intermediate step in defining ComboCellBody (see Supplementary Methods).

Figure 8. Time resolved multiexperiment analysis of receptor (PTH₁R and β_2 -AR), β -arrestin2, and colocalized object formation. Receptor, arrestin, and colocalization objects were identified using the segmentation demonstrated in Figure 2. Colors correspond to object type: receptor (green), arrestin (red), colocalizations (orange). The number of cells and transfections are noted in the text, but every series is represented by at least five cells from at least four separate transfections. Panels A–C are comprised of data obtained from cells transfected with YFP-tagged Class A GPCR β_2 -AR stimulated with agonist isoproterenol (Iso) with or

without β_2 -AR-specific antagonist ICI-118,551 (ICI) pretreatment as indicated in the figure legends. Panel **D** provides an overlay of receptor, arrestin, and colocalized object counts for Class A. Panels **E–F** are comprised of data obtained from cells transfected with YFP-tagged Class B GPCR PTH₁R stimulated with agonist hPTH_{1–34} with or without antagonist hPTH_{7–34}. Panel **H** provides an overlay of all Class B object counts. Panels **I–K** provide a comparison between Class A and Class B by each individual object type. Data shown are mean count of objects per cell among cells receiving same treatment. In panels **I–K** bars represent SEM calculated among cells per treatment group and receptor class.

Figure 9.

Class A and Class B GPCRs diverge in rate of colocalization with β -arrestin 2. Panels **A** and **D** display plots of the linear portions of (1) the initial formation of receptor objects and (2) the initial decay following maximum object counts of each individual object (receptor, β -arrestin 2, foci of colocalizations). Counts were averaged and linear regression of each of the two segments for each object was performed in Prism. Solid lines correspond to initial rates (V_i) and dashed lines correspond to post-maximal rate (V_{pm}). Panels **B**, **C**, **E**, and **F** plot the rates (object count per minute) $\pm$ SEM as bars for clarity. In panels **B** and **E**, linear

regression analysis was performed to compare the slopes of objects within each GPCR class (using the rate of receptor object formation within each class as reference) and statistically significant changes noted. In panels **C** and **F**, a similar regression analysis was performed, instead using zero as the referent slope (a zero slope indicates persistence of object counts over time). * $p < 0.006$; ** $p < 0.0001$.

Figure 10.

Time resolved multiexperiment analysis of receptor (PTH₁R and β_2 -AR), β -arrestin2, and colocalized object counts per cell. Individual cells were defined in Spotfire by each unique LinkedTrackID per Field per Well. Counts are plotted versus time. Following selection criteria outlined in the methods, a total of five cells were analyzed for the prototypical Class A GPCR β_2 -AR, and six cells were analyzed for the Class B GPCR prototype PTH₁R.

Figure 11.

Time resolved multiexperiment analysis of receptor (PTH₁R and β_2 -AR), β -arrestin2, and colocalized object counts in cells that received no agonist (vehicle control). Counts are plotted versus time. The value of 20 detected β -arrestin2 objects detected on average in the Class A dataset was used to determine the levels for noise in this assay. Counts averaged over the cells and reported here $\pm$ SD among cells.

Figure 12.

Time resolved multiexperiment analysis of receptor (PTH₁R and β_2 -AR), β -arrestin2, and colocalized object area (averaged per cell). Area of each object was determined and then summed to determine total area within each cell. These total areas per cell were then averaged over the cells and reported here $\pm$ SD among cells.

Figure 13.

Time resolved multiexperiment analysis of receptor (PTH_1R and $\beta_2\text{-AR}$), β -arrestin2, and colocalized object size (averaged per object). Total area of each object was determined per cell and then divided by total count per cell to determine average size of object. These average object sizes per cell were then averaged over the cells and reported here $\pm$ SD among cells.

Figure 14.

Time resolved multiexperiment analysis of cell morphology. Average Area and Form Factor [defined in equation (1)] of all cells per transfection, under agonist or vehicle conditions. Form Factor is a measure of complexity, varying from 0 (irregular) to 1 (a perfect circle). Error bars are $\pm$ SD among cells.

Figure 15.

Time resolved multiexperiment analysis of cell movement. Cellular position within the microscopic field was simulated by plotting y position versus x position (both in μm , "0" is referent to bottom left pixel). Each color represents an individual cell as determined by unique LinkedTrackID per well per field and lines connect each unique cell. All fields are superimposed upon one another and colors correlate with Figure 10.

Objects Identified (Count)

Figure 16.

Correlation between counts of receptor, β -arrestin2, and colocalization objects and cell size displays divergence between Class A and B GPCRs. Heterogeneity of cellular responses to ligand is in part explained by the size of the cell. Points are colored on a per cell basis, and all points associated with a single cell linked to each other by one line. Directional arrows demonstrate order of time. Larger cells (toward the right) correlate positively with the total number of receptors detected. Regression lines (thick black line) were calculated in GraphPad Prism and plotted manually atop Spotfire-generated graphs using Adobe Illustrator.

Table 1

Summary of Quality Control.*

Dataset (Treatment + Pretreatment)	Number of Cells	Number of Targets (Cells and Junk)	Inclusion %	Number of Wells	Number of Transfections
Class A – YFP-β ₂ -AR					
Isoproterenol 1 μM	14	24	58.3%	5	3
Isoproterenol 10 nM	20	28	71.4%	5	3
Isoproterenol 10 nM + ICI 118,551 1 μM	11	25	44.0%	3	3
Vehicle	5	26	19.2%	4	3
Class B – YFP-PTH ₁ R					
hPTH ₁₋₃₄ 1 μM	9	21	42.9%	4	3
hPTH ₁₋₃₄ 10 nM	5	20	25.0%	3	3
hPTH ₁₋₃₄ 10 nM + hPTH ₇₋₃₄ 10 μM	6	17	35.3%	5	5
Vehicle	9	25	36.0%	4	2
Overall					
All datasets	79	186	41.5%	33	25

* The quality control process—outlined in the Data Analysis section of the Methods and exemplified for one microscopic field in Figure 3—was conducted for all 33 fields and tabulated here for a quantitative assessment of how many cells were of high enough quality for inclusion into the dataset. As you can see, there were at least three biological replicates (as evidenced by number of wells) for all datasets.