Rapid Selection and Development of GPCR expressing Mammalian cell lines using novel ClonePix Technology

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Overview

The G-protein-coupled receptor (GPCR) family represents the largest and most versatile group of cell surface receptors. GPCRs play a pivotal role in cell signaling and are targets of approximately 50% of all pharmaceutical drugs in the marketplace. Identifying key modulators of GPCRs is of critical interest for drug discovery and the development of cell based screening technologies aimed to identify novel drugs against both known and orphan GPCRs are of utmost significance. The endogenous expression of GPCRs in mammalian cells is typically very low with generally no more than 3,000 copies per cell and thereby present a challenge with respect to GPCR drug discovery efforts. Most screening assays require a much higher concentration of functional GPCRs presented on the cell surface. A critical bottleneck in GPCR studies is the difficulty of expressing soluble and stable receptors in sufficient quantities. Attempts to create expression systems in "lower" organisms have met with limited success due to inefficient folding (bacteria), low yield (yeast) or incorrect post-translation modification (baculovirus). These challenges fuel the market need for mammalian expression systems capable of providing the elevated GPCR protein expression levels required to support drug discovery efforts. Discovery and selection of high expressing GPCR clones from a transfected pool of cells can be challenging with regards to cell-line development. We present here a one-step solution and automated high-throughput platform technology that would aid in the identification and isolation of rare clones from large heterogeneous pool of transfected cells. Utilizing both white light and fluorescent based imaging in situ, the clone selection system has both the sensitivity and specificity to quantitatively detect the endogenous and cell surface protein expression levels of respective GPCRs. This eliminates the need for labor- and time-intensive manual methods such as limiting dilution. The fluorescent based automated system can thus be efficiently used to detect and pick respective GPCR expressing clones, and thereby provide as a unique source of high quality GPCR proteins for variety of applications, including antibody generation using antigens with natural epitopes at high expression level and cell-based functional assays for hard-to express GPCRs from structurally distinctive GPCR families.

Introduction

The endogenous expression of GPCRs in mammalian cells is typically very low, generally with no more than 3,000 copies per cell. These low endogenous expression levels present a challenge with respect to GPCR drug discovery efforts as most screening assays require a much higher concentration of functional GPCRs. Mammalian expression systems capable of providing the elevated GPCR protein expression levels would provide a solution to some of the challenges in drug discovery and cell line development.

The technology in ClonePix Systems represents a proven, one-step method of screening large heterogeneous cell populations rapidly (10,000 clones in 3 weeks) increasing the probability of finding optimal producers due to the significantly larger pool of cells that can be analyzed. Utilizing both white light and fluorescent images in situ, the ClonePix 2 System has the sensitivity to detect endogenous levels of protein limited to cell surface expression.

A transfected CHO-M1 cell line expressing endogenous muscarinic 1 cholinergic receptor (GPCR – M1) was chosen to demonstrate the feasibility of using the ClonePix 2 System to detect cell surface expressed proteins. CHO-M1 expressing clones were screened using anti-M1 antibody conjugated with PE (Phycoerythrin), and selected based on fluorescence intensity and picked using the ClonePix 2 System.

Methods

Growth of GPCR M1 expressing cell line in Semi-Solid Media
A transfected CHO-M1 cell line expressing endogenous muscarinic 1 cholinergic receptor (GPCR – M1) was chosen to demonstrate the feasibility of using the ClonePix 2 System to detect cell surface expressed proteins. CHO-M1 and parental untransfected CHO-K1 cells (negative control) were plated in CloneMedia CHO (Molecular Devices K8710) semi solid media at low density and incubated at 37ºC for 8–10 days until discrete colonies were formed.

Screening of GPCR M1 with fluorescent labeled anti-M1 antibody
Both a direct labeled and dual-label approach were tested by adding antibody along with cells in semi-solid medium. For the direct labeled approach, a PE-labeled anti-M1 antibody was used. For the dual-label approach, an unconjugated rabbit anti-M1 antibody and a PE-labeled anti-rabbit polyclonal antibody were used. Control wells containing cells without antibody were also plated.

Imaging and picking of GPCR (M1) expressing clones using the ClonePix 2 System
Both CHO-M1 and CHO-K1 cells were imaged and picked using the ClonePix 2 System. Brightfield cell imaging was used to identify the morphology and location of each colony, and fluorescence to identify the highest expressers of M1. CHO-M1 cells expressing M1 GPCR produced a range of fluorescent signals on the ClonePix 2 System with both the directly labeled antibody and dual-antibody approach. The fluorescent signals recorded correspond to the various levels of expression of the M1 GPCR. The parental line CHO-K1 did not produce a fluorescent signal.

Instrument overview

ClonePix 2 System
- Automated system for screening and selection of mammalian clones
- Images using white light and fluorescence
- Software-controlled switching between up to 5 excitation / emission filter pairs
- Ranks colonies using user-definable criteria
- Target colonies identified and picked into 96 well destination plates
- Supports multiple applications for both suspended and adherent cell lines - Screening / selection of hybridomas secreting antibodies and Cell line development

CloneSelect Imager
- Label-free white light imaging of cells
- Objective, quantitative assessment of cell growth
- Simple, user-friendly software interface
- Supports multiple applications including:
  - Monitor cell growth rates
  - Verification of monoclonality

FLIPR Tetra System
- Standard EMCCD fluorescence or optional ICCD fluorescence and luminescence detection
- User-configurable 96-, 384-, and 1536-well pipettors
- User-exchangeable cell suspension option
- Unique, configurable excitation optics for expanded dye capabilities
- Intuitive, user-friendly software interface
- TetraCycler Internal Plate Handler for accelerated throughput

Results: Detection of CHO-M1 cells on ClonePix 2 System

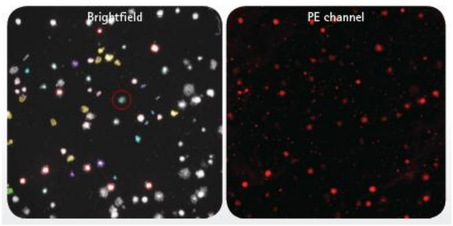


Figure 1. The ClonePix 2 System reveals diverse levels of fluorescent intensity with CHO-M1 cell line, demonstrating it can distinguish between various levels of expression of GPCR M1 protein. Colonies recognized by the software are outlined in color under the brightfield channel. Fluorescence intensity is calculated based on the physical location of colonies.

Results: Fluorescent ranking of CHO-M1 expression

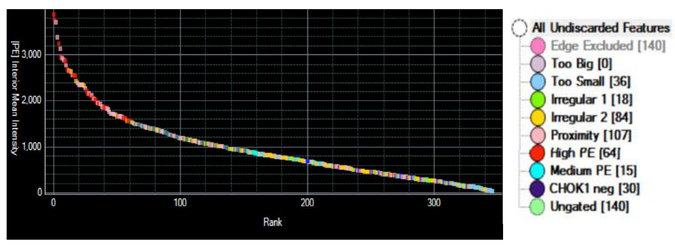


Figure 2. Clones ranked and grouped based on morphology and interior mean fluorescence intensity. Acceptable selection criteria for picking includes size, shape, and proximity to neighboring colonies. Clones are ranked by interior fluorescent intensity and gated into one of four fluorescent groups: High, Medium, CHO-K1 neg, and Ungated.

Results: Image Analysis and Custom Clone Selection

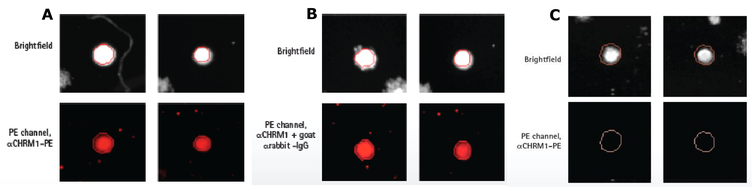
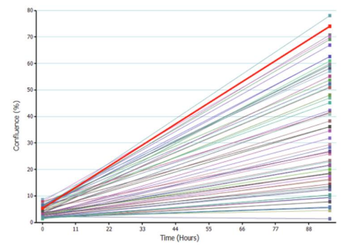


Figure 3. Selection of GPCR M1 expressing clones with both direct labeled antibody (A) and dual labeled antibodies (B) approach are shown (brightfield and fluorescence images). The fluorescent intensity is proportional to M1 expression in the positive clones. The negative control, CHO-K1 cell line demonstrates no fluorescence in the PE channel (C).

Results: Growth curve of ClonePix picked cells



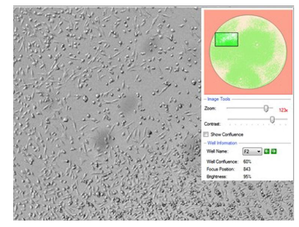


Figure 4. ClonePix picked clones were deposited in 96 well tissue culture plates and imaged on CloneSelect Imager to verify transfer and subsequent growth post-culturing for 1 week. Growth curve generated by CloneSelect Imager demonstrated proliferation of picked cells. Confluence is automatically calculated by the software.

Results: Functional validation of picked cells with FLIPR Tetra System and FLIPR Calcium 6 Assay Kit

The results are depicted here: (A) CHO-M1 high, (B) medium to low M1 fluorescence, and (C) CHO-K1 negative control picked groups using the ClonePix 2 System and evaluated for functional activity. The FLIPR Tetra System performs high-throughput, functional cell-based assays and is the system of choice in drug discovery for evaluating changes in intracellular calcium detected through the use of fluorescent calcium-sensitive reporter dyes(FLIPR Calcium 6 Assay Kit). The changes in cytoplasmic calcium via activation of the G-protein coupled IP3 sensitive pathway by carbachol was assessed at 40 nM.

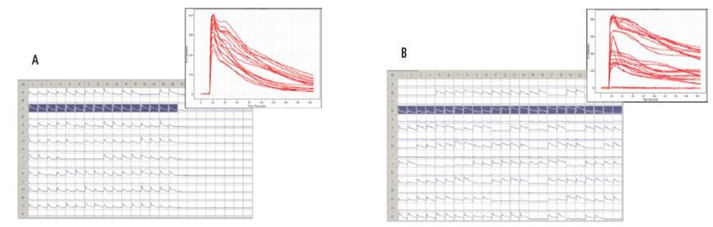


Figure 5. In the CHO-M1 high fluorescent picked clones, carbachol produced a four-fold increase in fluorescent read from background (Figure 5A). In the mixed CHO-M1 medium and low fluorescent picked clones, carbachol produced a four- and two-fold increase in fluorescent read from background respectively (Figure 5B).

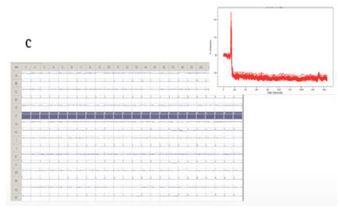


Figure 5C. In the CHO-K1 negative group, carbachol failed to elicit any significant change in fluorescent read from background.

These results support a positive correlation between membrane bound M1 GPCR expression level and functional activity. The lack of calcium fluorescent signal in CHO-K1 negative control group further confirms that the ClonePix 2 System can accurately distinguish between clones with and without expression of M1 GPCR on the cell surface.

Conclusions

- The ClonePix 2 System can reliably detect variable expression of GPCR clones and is an efficient means for high-throughput selection of clones of interest.
- Utilizing both white light and fluorescent images *in situ*, the ClonePix 2 System has the sensitivity to detect endogenous levels of protein limited to cell surface expression.
- The ClonePix 2 System can thus be efficiently used to detect and pick respective GPCR expressing clones, and provide high quality GPCR proteins for variety of applications.

- Automated system for screening and selection of mammalian clones of interest.
- Supports applications for both suspended and adherent cell lines
- Simple, user-friendly software interface.
- Imaging with white light and fluorescence.
- Ranking colonies using user-definable criteria.

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