

APPLICATION NOTE

Rapid selection and development of GPCR expressing mammalian cell lines using novel ClonePix Technology

ClonePix™ 2 Systems provide a new method to rapidly assess GPCR target protein expression levels in mammalian cell lines.

Background

The endogenous expression of GPCRs in mammalian cells is typically very low, generally with no more than 3,000 copies per cell. These endogenous expression levels are sufficient to maintain proper receptor function but 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. For example, structural studies, small molecule drug design and generation of functional antibodies against native GPCR targets require expression levels that are orders of magnitude higher than endogenous levels. 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.

Benefits

- Rapidly assess GPCR target proteins at endogenous levels using mammalian expression systems
- Increase probability of finding optimal producers
- Cut cell line/antibody development times – avoid limiting dilution

Discovery and selection of high expressing GPCR clones from a transfected pool of cells can be challenging with regards to cell-line development. The technology in ClonePix 2 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.

Methods

A transfected CHO-M1 cell line expressing endogenous G-protein coupled 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), selected based on fluorescence intensity, and picked using the ClonePix 2 System. The parental CHO-K1 (wild-type) cell line was used as a negative control. Colonies picked by the ClonePix 2 System were monitored for proliferation by CloneSelect™ Imager, a label-free system for objectively assessing cell growth. Functional validation of clones isolated using the ClonePix 2 System was performed using the FLIPR® Tetra High-Throughput Cellular Screening System and FLIPR® Calcium 6 Assay Kits from Molecular Devices. 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 response following GPCR activation as detected through use of fluorescent calcium-sensitive reporter dyes.

Materials

- Cell line: CHO-M1 cell line expressing endogenous G-protein coupled muscarinic acetylcholine receptor M1 (M1 WT3) (ATCC, Cat. #CRL1985). Parental CHO-K1 cells used as negative control.
- Antibodies: Rabbit Anti-ChRM1-PE labeled, polyclonal (Bioss, Cat. #ABIN668656); Rabbit Anti-ChRM1 polyclonal Abs, unconjugated (Bioss, Cat. #bs-1150R); Goat antirabbit IgG - PE labeled, polyclonal (Life Technologies, Cat. #P2771MP).
- Culture Media: Ham's F12 media (Corning, Cat. #10-080-CV); CloneMedia-CHO-S (Molecular Devices, Cat. #K8710); Fetal Bovine Serum (Hyclone, Cat. #SH30071.03), 1% Pen/Strep/Glutamine (Life Technologies, Cat. #10378016), 450 µg/mL Geneticin, G418 (Life Technologies, Cat. #10131035)

- Assay Buffer: 10X HBSS (Life Technologies, Cat. #14065056) diluted in sterile water for injection (Irvine Scientific, Cat. #9309), with 20 mM HEPES (Life Technologies, Cat. #15630080). Adjust pH to 7.4.
- FLIPR® Calcium 6 Assay Kits (Molecular Devices, Cat. #R8190)
- Plates: 6-well non-TC treated, clearbottom plates (Greiner Bio-One, Cat. #657185); 96-well clear F-bottom plates (Greiner Bio-One, Cat. #655185); 384-well black-wall, sterile, TC-treated, clear-bottom plates (Corning, Cat. #3072)
- M1 AChR agonist: Carbamoylcholine chloride or Carbachol (Sigma, Cat. #C4382)

Instrument overview

ClonePix 2 System

- Automated system for screening and selection of mammalian clones
- Images using white light and fluorescence
- Trans-illumination supports imaging low contrast colonies such as adherent monolayers
- 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 studies and thereby was used to screen novel subclones secreting high affinity antibodies.
- Supports multiple applications for both suspended and adherent cell lines including:
 - Screening and selection of hybridomas secreting antibodies of interest
 - Cell line development

CloneSelect Imager

- Label-free white light imaging of cells
- Objective, quantitative assessment of cell growth
- Simple, user-friendly software interface
- Growth rates accurately determined in every well of a 96-well plate

- Supports multiple applications including:
 - Monitoring cell growth
 - 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 with robotics

Plating of cells in semi-solid medium

CHO-M1 and CHO-K1 cell line: Both CHO-M1 and parental CHO-K1 cells were plated in CloneMedia CHO at a final density of 1,000 cells per well and incubated at 37°C for 8–10 days until discrete colonies were formed. Both a newer and older passage of CHO-M1 cells were plated to look for various levels of M1 GPCR expression.

Direct labeled approach: An antibody against the Muscarinic 1 Receptor (CHRM1) labeled with PE was added directly to the semi-solid medium along with the cells. Control wells containing cells without antibody were also plated.

Dual-label approach: In circumstances where a directly labeled antibody may not be available, the feasibility of using a primary and secondary antibody was tested. Both an unconjugated rabbit anti-muscarinic antibody and a PE-labeled anti-rabbit polyclonal antibody were added directly to the semi-solid medium along with the cells. Control wells containing cells without antibody along with wells containing only the secondary antibody were also plated.

Imaging and picking of GPCR (M1) expressing clones using the ClonePix 2 System

Both positive (CHO-M1) and negative (CHO-K1) cells were imaged and picked using the ClonePix 2 System. Cells were imaged in brightfield to identify the location and morphology of each colony and fluorescence to identify the highest expressers of M1. For the PE labeled probe, the Cy5 channel was used with a 6,000 ms exposure time.

CHO-M1 positive cells produced a range of fluorescent signals as recorded on the ClonePix 2 System with both the direct labeled antibody and the dualantibody approach. This corresponds to various levels of expression of the M1 GPCR (Figure 1). As expected, the parental line CHO-K1 did not produce a fluorescent signal.

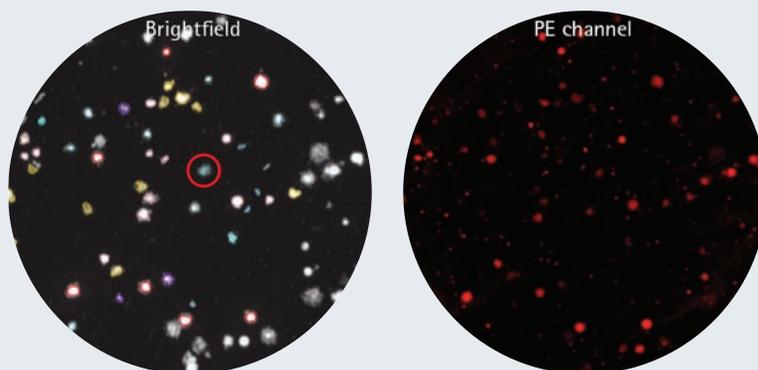


Figure 1. Detection of CHO-M1 cells on ClonePix 2 System. The ClonePix 2 System reveals diverse levels of fluorescent intensity with CHO-M1 cell line, demonstrating it can distinguish between various levels of expression of GPCR M1 protein. Colonies recognized by the software are outlined in color under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

Cells were grouped to identify and separate colonies based on morphology and fluorescent intensity. Acceptable morphology for picked colonies was based on size, shape, and proximity to neighboring colonies. Morphologically ideal colonies were ranked by interior fluorescent intensity and gated into one of four fluorescent groups: High, Medium, CHO-K1 neg, and Ungated (Figure 2). The CHO-K1 neg group was defined by intensity of background signal. All colonies identified in the group were confirmed to originate from the corresponding negative control well. Ungated colonies refer to colonies that had low levels of signal but above the background.

Both the direct labeled antibody and the dual-antibody approach (Figure 3) demonstrated fluorescent signal in positive samples (expressing M1), and lack of signal in the parental cell line (Figure 4). As expected, the dual-label approach produced more background signal in the PE channel due to primary and secondary antibody binding in the background. However, the ClonePix 2 System detects colonies in brightfield, then looks for fluorescent signal within the colony. Objects recognized in the PE channel but not in brightfield are not considered colonies; thus, the ClonePix 2 System provides the benefit of the ability to discriminate true colonies from the background signal.

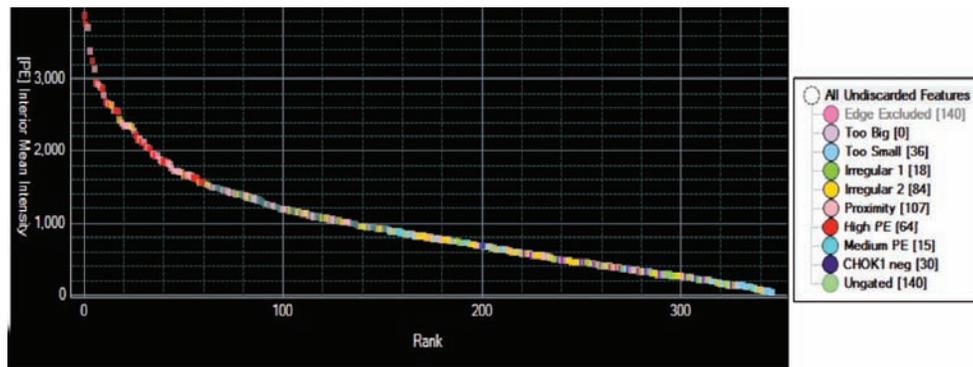


Figure 2. Fluorescent ranking of CHO-M1 expression. Clones were ranked, then grouped based on morphology and interior mean fluorescence intensity.

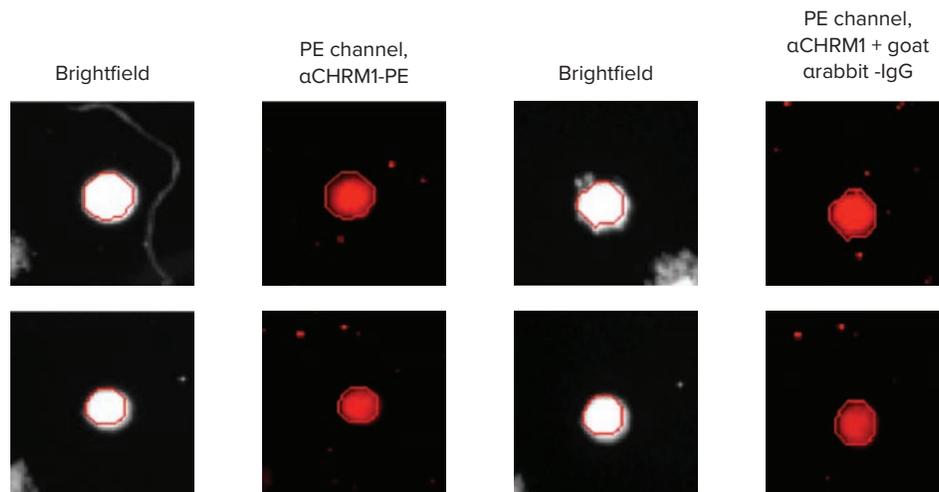


Figure 3. CHO-M1 positive cells imaged using ClonePix 2 System. Fluorescence Based Image Analysis and Custom Clone Selection using ClonePix 2 System. GPCR M1 Positive clones images are shown above. Selection of M1 expressing colonies with both direct labeled antibody and dual-labeled antibodies approach are shown.

Colonies picked by the ClonePix 2 System were deposited into 96-well plates containing 200 μ L Ham's F12 media + 10% FBS + G418 for CHO-M1 picked clones and the same media conditions without G418 for CHO-K1 clones. These 96-well plates were imaged on the CloneSelect Imager to verify transfer and growth (Figure 5). Cells were cultured

for one week then analyzed on the CloneSelect Imager to ensure the cells had proliferated (Figures 5 and 6). Cells were subsequently transferred to 384-well plates for functional verification of expression by the FLIPR Tetra System using the FLIPR Calcium 6 assay.

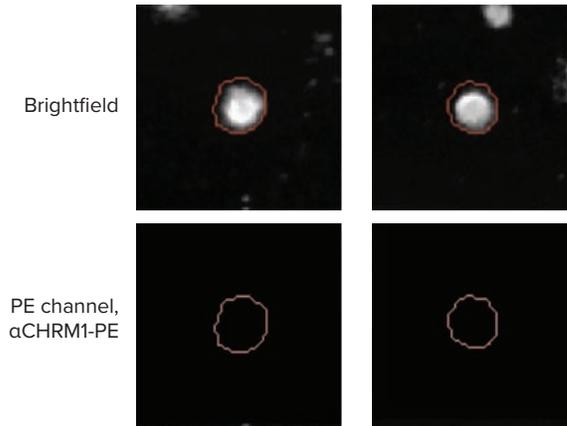


Figure 4. CHO-M1 negative cells. Parental CHO-K1 cell lines (negative control), PE-Fluorescence and brightfield images of picked clones.

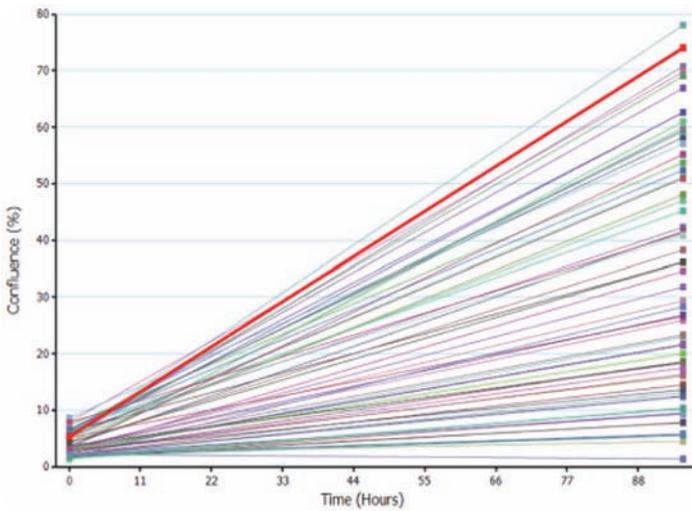


Figure 5. Growth curve of CHO-M1 cells. Growth curve generation by the CloneSelect Imager demonstrating proliferation of picked cells.



Figure 6. Cells imaged with CloneSelect Imager. Image of CHO-M1 cells acquired on the CloneSelect Imager. Confluence is automatically calculated by the software.

Validation of M1 GPCR expression in picked cells by FLIPR Tetra System

Cell preparation

Cells selected by the ClonePix 2 System were plated on a 384-well plate overnight at 5,000 cells per 25 μ L of growth medium per well and incubated at 37°C in 95% humidity and 5% CO₂. These cells were sorted into four groups: high, medium, low, and no M1 expression based on the ClonePix System technology.

Calcium-sensitive fluorescent dye loading

Cell plates were removed from the incubator and equilibrated to room temperature before dye loading. 25 μ L of FLIPR Calcium 6 Dye was added to cell plates without removing the culture media. These plates were incubated for two hours at 37°C in 2.5 mM probenecid to block organic anion transporter and then kept at room temperature until fluorescent read.

FLIPR Tetra Fluorometric Imaging Plate Reader

After incubation and equilibration to room temperature, one plate was placed on the plate reader platform of the FLIPR Tetra System. Fluorescent calcium signal was captured by an ICCD camera with the following parameters:

- Exposure (sec): 0.53
- Excitation LED (nm): 470–495
- Emission Filter (nm): 515–575
- LED Intensity: 80

FLIPR Calcium 6 Assays

Fluorescent readings were taken once every second; 10 and 60 reads were taken before and after 40 nM carbachol addition, respectively. Starting carbachol concentration was set to 5-fold greater than the final concentration. Carbachol addition volume was 12.5 μ L and dispensed at 20 μ L/sec.

Validation of picked CHO-M1 and CHO-K1 clones using the FLIPR Calcium 6 Assay Kit

Membrane bound G protein-coupled muscarinic receptor expression level was evaluated using anti-M1 antibody conjugated with PE from a newer and older passage of CHO-M1 cells. The expectation is that with the older passage the expression level of M1 GPCR will be significantly lower than the newer passage. Parental CHO-K1 cells were also included in the study as negative control.

Upon activation of GPCRs by ligands, receptor conformation is changed triggering G-protein activation inside the cell. An active G-protein has the potential to induce various cascades of intracellular messengers including calcium.

Cells from each group picked by the ClonePix 2 System were evaluated for functional activity using the FLIPR Tetra System. Calcium-sensitive fluorescent dye (FLIPR Calcium 6 Assay Kit) was used to assess changes in cytoplasmic calcium via activation of G-protein coupled IP3 sensitive pathway by carbachol at 40 nM throughout this feasibility study. Carbachol at 40 nM is the EC₈₀ concentration based on historic agonist concentration response curves, determined empirically.

In the CHO-M1 high fluorescent picked clones, carbachol produced a four-fold increase in fluorescent read from background (Figure 7A). 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 7B). Finally in the CHO-K1 negative group, carbachol failed to elicit any significant change in fluorescent read from background (Figure 7C). Since each group, except the mixed medium and low fluorescent picked clones, was seeded on a separate 384-well plate, changes in baseline fluorescence intensity were normalized to background fluorescent reads of each 384-well plate before the addition of 40 nM carbachol.

These results support a positive correlation between membrane bound G-protein coupled muscarinic receptor 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 the M1 GPCR on the cell surface.

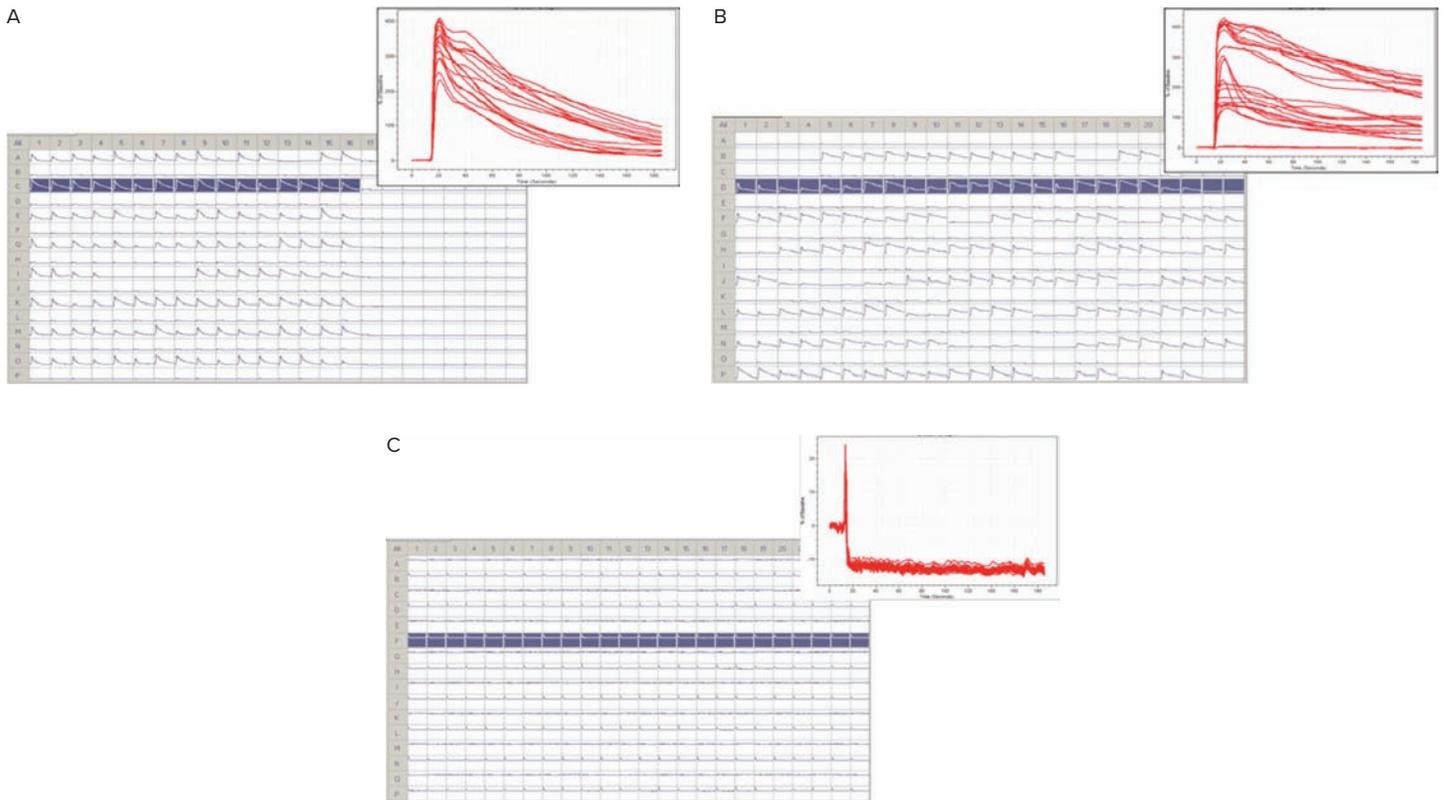


Figure 7. Analysis on FLIPR Tetra System. The FLIPR Tetra System performs high-throughput, functional cell-based assays and is the system of choice in drug discovery for evaluating changes in intracellular calcium detected through the use of fluorescent calcium-sensitive reporter dyes (FLIPR Calcium 6 Assay Kit). The results are depicted here: (A) CHO-M1 high, (B) medium to low M1 fluorescence, and (C) CHO-K1 negative control.

Conclusion

To meet the demands of high-throughput screening and selectivity profiling, GPCR transfected cell lines are a unique, robust, and uniform platform for cell-based functional assays. In conclusion, these preliminary studies revealed that the ClonePix 2 System can reliably detect variable expression of GPCR clones. Moreover, fluorescence intensity correlates positively with the magnitude of GPCR-mediated changes in cytoplasmic calcium levels as measured by the FLIPR Tetra instrument, caused by differences in the expression of membrane bound G-protein coupled muscarinic receptor M1.

The ClonePix 2 System can thus be efficiently used to detect and pick respective GPCR expressing clones and 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.

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