High Throughput Screening and Selection of Clonal Populations of Reporter Cell Lines, using Fluorescence Cell Based Assay Techniques

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Introduction

Advances in cell culture techniques are facilitating development of novel screening assays which enable drug discovery programmes to rely on cell-based assays for high-throughput screening in lead compound identification and development projects. Many such cellular assays utilise fluorescent readouts for measuring cell viability, cytoxicity, apoptosis, response to stimuli or induction of reporter genes/pathways.

For all such cell based assays it is essential that the cell line being used is as robust and homogeneous as possible and that any transfected producer/reporter constructs are stable. Reporter cell lines should also be selected for their signal-to-noise ratio and sensitivity to ligand, etc.

Here, we show how Genetix technology in fluorescent imaging and picking colonies of clonal cells, from mixed populations, can be used to remove bottlenecks in selecting both high and stable producer cell lines, for therapeutic protein secretion, high expressors of target constructs, and in the engineering of stable cell lines for cell based assay screens.

Therapeutic Protein and Antibody Production

Our established ClonePix^{FL} technology allows colonies of cells to be selected that are high producers and secretors of therapeutic protein. The ability of our technology to select colonies of CHO, NS0 and hybridoma cells secreting human or mouse IgG molecules, using fluorescently labelled anti-IgG probes (Figure 1), is the basis of our new range or assays, and uses the same fluorescent imaging and robotic processing and picking techniques.

The ClonePix^{FL} Technology

Genetix ClonePix^{FL} technology is based on the ability to grow mammalian cell lines in semi-solid media so that the cells form discrete clonal colonies, originating from one single parent cell. The colonies of cells can then be imaged using fluorescent detection reagents to identify colonies with a desired phenotype, for example:

- Fluorescently labelled anti-IgG probe for IgG secretion assays
- Conjugated antibody for cell surface or secreted protein
- Cellular reporter dyes for cell viability, apoptosis, ion channel function
- Reporter gene substrate dyes such as CCF-4 for β-lactamase assays

Once imaged, using white-light and/or up to five fluorescent wavelengths in one screen, colonies are analysed by our dedicated software and automatically picked by ClonePix^{FL}. Each colony is then dispensed into a media-containing well of a 96-well plate.

These plates of isolated colonies can then be grown to confluence in micro-titre plates before scaling up for use as producer cells or in HTS, whereby the population in each well will be a monoclonal population of cells; so insuring homogeneity as well as being positive for the desired fluorescent readouts.

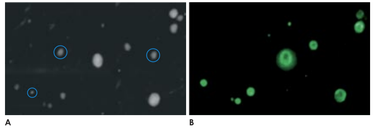


Figure 1: IgG secretion assay and colony detection. Colonies of CHO-S cells (previously transfected with human IgG construct) were grown in Genetix CloneMatrix semi-solid media, in black sided 6-well culture plates, and imaged on day 10 with ClonePix^{FL}. A) White-light image of all colonies within a picking region, with non-producer colonies (by fluorescence) highlighted with blue circles. B) FITC excitation/emission fluorescent image of the same colonies, showing detection of secreted IgG (with Genetix CloneDetect IgG detection reagent) around the more productive clones.

This has now been successfully extended to include the selection of hybridoma cells secreting IgG with a desired specificity (using fluorescently labelled antigen) and to the selection of transfected CHO and HEK293 cells secreting monomeric therapeutic proteins, using target-protein specific, fluorescently labelled antibody, as shown in Figure 2.

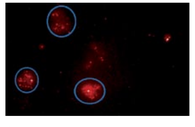


Figure 2: Detection of therapeutic protein secretion. Adherent HEK 293 cells stably transfected with a monomeric human protein of therapeutic interest: Cells were seeded at low density into 6-well tissue-culture treated plates under CloneMatrix-based semi-solid media, to form adherent clonal colonies, and imaged on day 13 with ClonePix^{FL} (with Rhodamine excitation/emission spectra). Image shows secreted protein detected with conjugated polyclonal antibody to the protein of interest – added by spraying onto the media at day 11. Blue circles indicate the highest producing colonies, with 'clouds' of secreted protein around the cells.

Multiplexing for Viability and Cytotoxicity in Cell Based/ Productivity Assays

The ability to simultaneously measure the viability of cells used in cell based assays, be they the secretion/production assays demonstrated previously, or reporter cell assays for screening, is of great benefit to the drug discovery process and to cell line engineering.

We have developed a fluorescent dye that stains only viable (living) cells for use in a range of cell based assays – LiveDetect^{Green} (Figure 3).

LiveDetect^{Green} is an acetoxymethyl based cytoplasmic dye that only fluoresces when absorbed into the cytoplasm of living cells, where it is metabolised to its fluorescent form. Cells can be imaged, in the FITC excitation/emission spectra, either as individual cells (in cell imaging technologies or epi-fluorescent microscopy) or as viable colonies of cells (for ClonePix^{FL} colony screening, as shown in Figure 3 & 4).

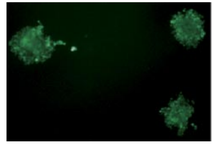


Figure 3: LiveDetect^{Green} viable cell and colony stain. CHO K-1 cells grown in CloneMatrix-based semi-solid media for seven days before addition of LiveDetect^{Green} reagent (by spraying). Colonies were then incubated for a further 2 hours to allow dye loading and metabolism to fluorescent form. Colonies imaged with ClonePix^{FL} using FITC excitation/emission filter sets at 10x magnification.

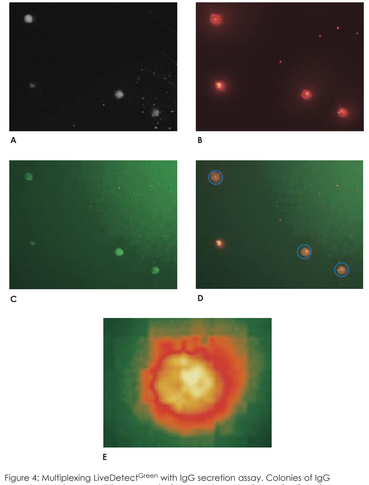


Figure 4: Multiplexing LiveDetect^{Green} with IgG secretion assay. Colonies of IgG secreting hybridoma cell lines grown in CloneMatrix semi-solid media. Colonies imaged with ClonePix^{FL} using: A) White-light control, B) IgG detection reagent using Rhodamine/PE filter set (IgG Secretion), C) LiveDetect^{Green} using GFP/FITC filter set (viability). Colonies which are both high producers of IgG and highly viable are indicated by blue circles on the merged image D. Panel E: merged image of a viable, secreting colony, imaged with our high-throughput fluorescent cell imager at 10X magnification, showing halo of secretion (red) around viable colony (yellow).

The advantage of multiplexing viability with other fluorescent readouts is that it gives much more power to a single assay or screen:

- Functional Screening – hit compounds simultaneously screened for cytotoxicity, allowing them to fail early if cytotoxic
- Cell line development – allows clones to be selected based on viability as well as production (Figure 4)
- Cell based assays – provides quality control of cells used in the assay, to ensure comparable viabilities across samples
- Apoptosis and ADMETox assays – confirmation of toxicity by simultaneous measurement of reduction in viability. In order to do this, we are developing a cellular dye that only stains dead cells and fluoresces in the Cy3™ excitation/emission spectra – allowing it to be multiplexed with LiveDetect^{Green} in toxicity studies, using fluorescent cell imaging platforms, shown in Figure 5

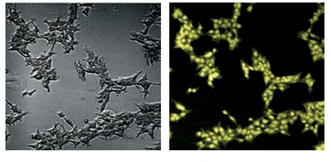


Figure 5: Fluorescence-based cytotoxicity assay. HEK 293 cells grown as a monolayer in a micro-titre plate, before being incubated in 50% ethanol for 30 minutes to desiccate the cells. Ethanol was then replaced with media containing Genetix prototype cytotoxicity stain and immediately imaged using our high-throughput cellular imaging technology. A) White-light image of cells after Ethanol treatment. B) Fluorescent image (in Cy3 excitation/emission spectra) showing uptake of cytotoxicity dye by damaged cells.

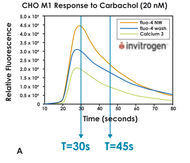
Cell Line Engineering for Reporter Cell Assays

• GPCR and calcium flux assay

We have now developed our imaging technologies to allow individual cells, or colonies of clonal cells to be imaged when containing reporter dyes for cell based assays, such as the calcium sensitive Fluo4-AM™ dye.

These developments allow more detailed screening of compounds by imaging individual cells as they respond to GPCR stimulation; the number of responsive cells can be quantified as well as the level of response from each cell. Using our high-throughput cell imaging system, we can clearly discern individual responsive cells from non responsive cells within the same population, as demonstrated in Figure 6.

It is also possible to grow such reporter cells into clonal colonies of cells that can then be imaged with our ClonePix^{FL} technology, having allowed the colonies to take up the reporter dye (Figure 6). This allows monoclonal cell lines (originating from one isolated, clonal colony) to be engineered that originate from the colony which (for example) had the highest signal-to-noise ratio when un-stimulated, or had the biggest increase in fluorescence when stimulated – making screening with such cells more sensitive and homogeneous.



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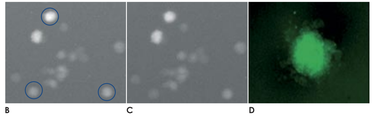

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Figure 6: Calcium sensitive GPCR reporter dye. A) Expected time-course for calcium response, as measured by Fluo-4 dye fluorescence intensity (from Invitrogen), indicating time-points at which images were taken. CHO K-1 cells were aliquoted into a 96-well micro-titre plate and loaded with Fluo4-AM dye as described by the manufacturer (Invitrogen). ADP, a P2Y GPCR agonist, was then added to the cells at a final concentration of 200nM and the cells we imaged, using Genetix high-throughput cellular imaging technology, at B) 30 seconds and C) 45 seconds after addition of ADP, to detect changes in fluorescence brought about by the associated Ca2+ Flux and subsequent re-setting (All exposure times = 5s). D) 10x magnification of a 15-day old colony of CHO K-1 cells, grown in CloneMatrix-based semi-solid media and loaded with Fluo4-AM for 6 hours prior to imaging on ClonePix^{FL}: showing background levels of detectable fluorescence with the FITC/GFP excitation/emission filter set.

• Reporter gene assays

As previously described for reporter dye assays, screens using reporter genes to provide fluorescent readouts, such as CCF-2/4 β-lactamase assays, also require sensitive, homogeneous reporter cells.

We have tested β-lactamase cell based assays on our high-throughput cell imaging system to show that we can image individual cells loaded with CCF-4 substrate and can clearly see the associated increase in fluorescence as the CCF-4 is metabolised from its negative (green fluorescent) to its positive (blue fluorescent) form, over time, by stably expressed β-lactamase – shown in Figure 7.

Developing this technology further will allow reporter gene cell lines to be engineered that are monoclonal and have more specific, sensitive or brighter response to positive control compounds for the pathway under investigation. Thus the sensitivity and homogeneity of the screen will be dramatically increased, making such campaigns more efficient, especially when combined (multiplexed) with our other fluorescent assays such as viability, toxicity, etc.

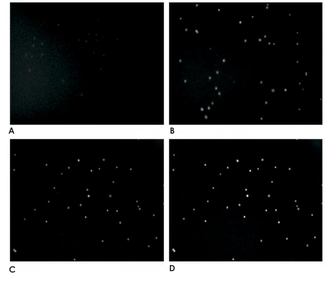

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Figure 7: β-Lactamase reporter gene assay. A) wtCHO K-1 cells imaged using DAPI/BFP filter set: showing background (negative) levels of fluorescence after 30 minutes incubation with CCF-4 β-lactamase substrate. B) CHO K-1 cells stably expressing the β-lactamase reporter gene (from a CMV promoter construct) imaged using DAPI/BFP filter set: showing (positive) levels of fluorescence after 30 minutes incubation with CCF-4, and C) After 45 minutes incubation with CCF-4. D) The same cells as in C, but after 80 minutes incubation with CCF-4. All images taken using Genetix high-throughput cellular imaging technology, at 2s exposure times.

Summary and Conclusions

Through expanding the applications for our cell and colony imaging technologies, we have identified new procedures for screening and selecting cells using fluorescent markers.

The ability to engineer and develop cell lines using these techniques allows cell lines to be optimised that are not only monoclonal, and so homogeneous, but are also selected for criteria such as viability, responsiveness to ligands (sensitivity), signal-to-noise ratio, etc. This has the potential to greatly improve the efficiency and effectiveness of cell based assays, removing bottlenecks and giving more reliable data from several readouts at once.

We have demonstrated that it is possible to screen and select clonal populations of cells for use in secretion of therapeutic antibody and monoclonal proteins, or as reporter cells in cellular dye (GPCR) assays and reporter gene assays. Such selection can be multiplexed with measurements of cell viability and toxicity for use in apoptosis assays or to allow cytotoxic compounds or unstable cell lines, to be excluded early in a screening campaign.

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