Transfluor® Assay on the iCyte™ Imaging Cytometer

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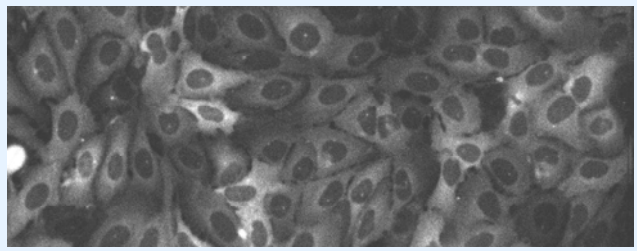
Introduction

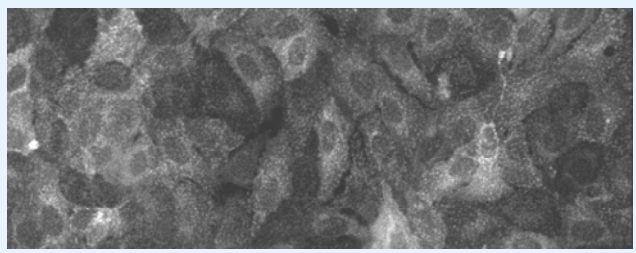
Transfluor® technology is a cell-based fluorescence assay system for screening G-protein coupled receptors (GPCRs) developed by Norak Biosciences (Morrisville, NC, USA)[1]. The Transfluor assay was developed based on the receptor desensitization mechanism. Upon ligand binding, GPCR is activated by association with G protein and then desensitized by phosphorylation and arrestin binding followed by internalization and recycling. The desensitization mechanism is common to most GPCRs. The Transfluor assay measures receptor activities by quantifying the redistribution of arrestin protein from cytosol to GPCR on the cell membrane and endosome. By labeling the arrestin molecule with green fluorescence protein (GFP), the Transfluor assay can visually monitor the processes of desensitization. Because the desensitization is closely coupled to activation of GPCR, changes in the intracellular distribution of the arrestin molecules reflect the active status of GPCRs. By using a cell line transfected with expression vectors of a particular GPCR molecule and the arrestin-GFP, it is possible to evaluate agonistic and antagonistic activity of chemical compounds towards specific GPCRs.

In the absence of agonist stimulation, arrestin-GFP molecules are evenly distributed throughout the cytosol (Figure 1A).

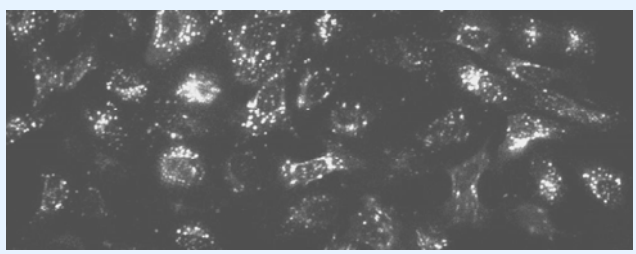
Once GPCR is activated, intracellular movement of the arrestin-GFP protein can be observed under a fluorescence microscope: toward clathrin-coated pit (Pit-type signals, Figure 1B) within seconds; or toward endocytic vesicles within minutes (Vesicle-type signals, Figure 1C).



1A. Unstimulated
U-2 OS cells co-expressing arrestin-GFP plus wild-type beta2-adrenergic receptor without receptor stimulation.



1B. Agonist-stimulated Pit-type signals
High-dose agonist-treated U2-OS cells co-expressing arrestin-GFP and wild-type beta2-adrenergic receptor.



1C. Agonist-stimulated Vesicle-type signals
High-dose agonist-treated U2-OS cells co-expressing arrestin-GFP and modified beta2-adrenergic receptor.

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Laser Scanning Cytometry

The iCyte™ Automated Imaging Cytometer, developed by CompuCyte Corporation (Cambridge, MA, USA) is designed to meet the demand in the biotechnology and pharmaceutical markets for automated high-content cellular analyzers which provide for higher throughput. iCyte has an architecture suitable for quantitative evaluation of characteristics of compounds measuring biological responses of cells and tissues. iCyte plays two roles: to acquire brightfield and fluorescent images of cells and tissues automatically; then, to convert molecular and cellular biological states to numerical data by analyzing these images within various parameters. These numerical data outputs are further subjected to processing by statistical analysis software to calculate numerical indexes for quantitative evaluation of chemical compounds.

Multi-well plates are often used to analyze cultured cells on iCyte. Because iCyte automatically captures cell images in each well, it is possible to track gradual changes of cellular responses corresponding to a dilution series of chemical treatment. Laser image scanning is carried out in a non-confocal manner. Brightfield images are acquired by detecting scattered light with a special detector, and fluorescent images are obtained by incorporating light emission detected by photomultiplier tubes (PMT). Although slower than some camera-based systems, this optical architecture is superior in terms of constituent quantitation.

The iCyte™ Automated Imaging Cytometer

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Materials and Methods

Test sample plates were obtained from Norak Biosciences for evaluating the Transfluor assay on the iCyte platform, and image analysis algorithms were designed for this combination.

Cell Preparation

A human osteosarcoma cell line U-2 OS stably expressing beta-arrestin-GFP together with either wild-type (Pit-type) or modified (Vesicle-type) beta2-adrenergic receptor was used. In the modified receptor, C-terminal amino-acid sequence was modified to convert the receptor from pit-type to vesicle-type. 1.7×10^4 cells were seeded in each well of a 96-well microplate and treated with various concentrations of isoproterenol (3.4 pM -200 nM, in three-fold dilutions) and fixed. Nuclear DNA was stained with DRAQ5™ (Biostatus, Leicestershire, UK). Sample preparation was carried out at Norak Biosciences.

Cytometer and Image Scanning

The iCyte™ Imaging Cytometer with iCyte Cytometric Analysis Software (V2.1.2) and iBrowser™ Data Integration Software (V2.1.2) was used for fluorescent image acquisition and data processing. GFP molecules were excited with an Argon ion laser (488 nm) and the green emission was acquired by a photomultiplier tube (PMT) fitted with a 515 - 545 nm bandpass optical filter. DRAQ5™ stained chromosomal DNA was excited with a Helium Neon (HeNe) laser (633 nm) and its long red emission was detected after passing through a 650 - 700 nm filter. A 40X objective lens was selected throughout the experiment.

Statistical Analysis

EC_{50} values were calculated by using GraphPad Prism® (GraphPad Software). Z'-factor was determined as previously described[4].

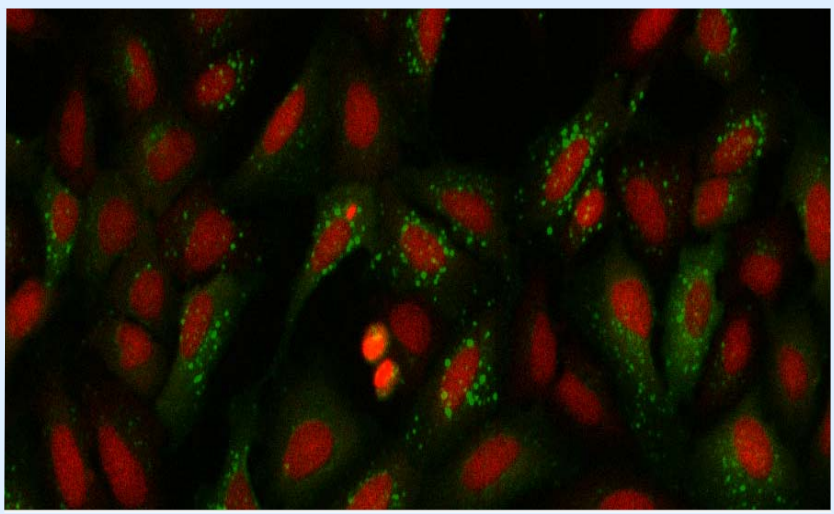
Image Analysis Algorithms

We tested two analysis algorithms to extract numerical data from acquired cell images: counting GFP signals and MaxPixel measurements. Although a number of fluorescence spots are seen on the Transfluor assay cells after agonist treatment, as a result of our preliminary data we concluded that it is not necessary to pick up information from all of these signals. Instead we chose small numbers of representative signals and converted their characteristics to numerical data.

Counting GFP Signals

Although both pits and vesicles were rich in the cytoplasmic area surrounding the nuclear area on the acquired cell images, the GFP signals tended to pile up to form high-density signal clumps that made it difficult to segregate individual spots. We found that we were able to avoid this problem by focusing exclusively on the nucleus. In the nuclear area, the cytoplasmic layer was thin and green signal density was relatively low, allowing each spot to be easily and precisely segregated from the others.

2A. Pseudocolored image

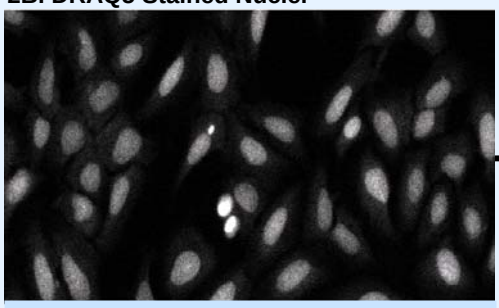


The iCyte pseudocolor feature highlights the long red (DRAQ5-stained nuclei) and green (arrestin-GFP) emissions.

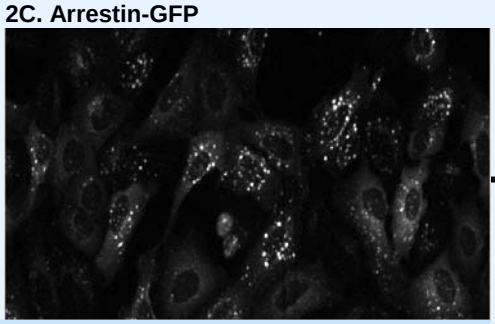
Analysis – Counting GFP Signals

Using the iCyte software, we contoured nuclear areas by selecting the long red emission image (Figure 2B), followed by setting an appropriate threshold level (the primary contour). Then we set another contour threshold for green emission (subcontour, Figure 2C) on the primary-contoured area (Figure 2D). Contoured green areas were sorted into four groups by using the gating function with the Area parameter (marked by colored boxes: green, red, yellow, and blue) (Figures 2E and 2F, following page). After processing, we obtained a certain number of representative GFP spots. Using the Area feature, we could choose particular spot groups that consisted of mainly pit-type or vesicle-type signals (Figure 2E).

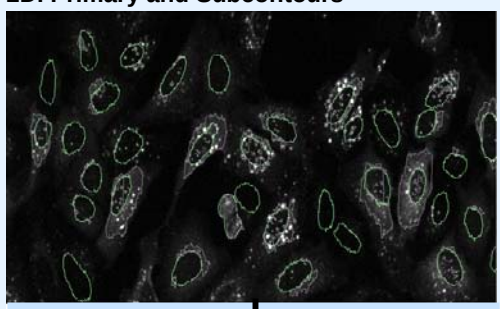
2B. DRAQ5 Stained Nuclei



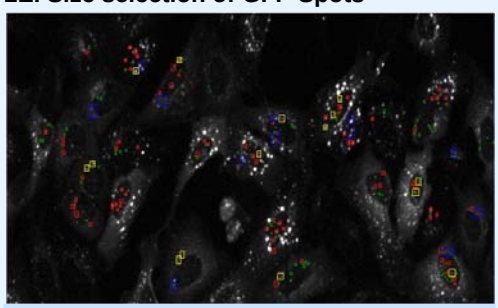
2C. Arrestin-GFP



2D. Primary and Subcontours



2E. Size selection of GFP Spots

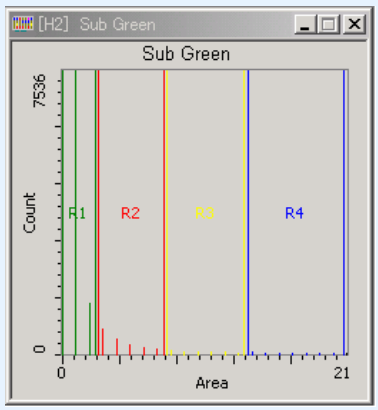


Analysis – Counting GFP Signals, continued

The gating and appropriate coloration are set in an iCyte histogram (Figure 2F).
For each Area range, iCyte calculated the number of green spots within each scanned area, as well as the primary-contoured signal number.

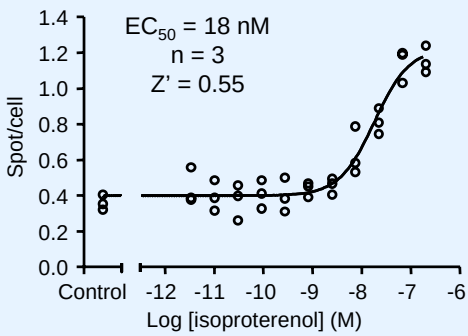
To normalize cell number differences among the wells, the green spot counts were divided by the corresponding primary-contour count for each well.

2F. iCyte Histogram



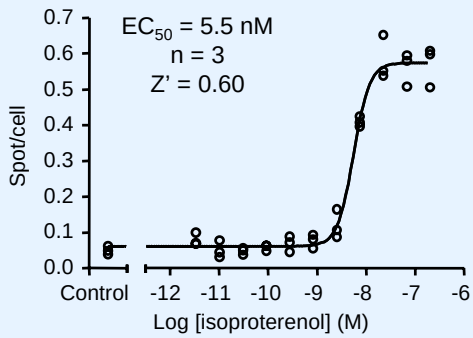
EC_{50} estimations, below, were performed by the statistical software.

3A. Wild-type β2AR (Pit-type signals)



U-2 OS cells expressing wild-type beta2-adrenergic receptor challenged with various concentrations of isoproterenol.

3B. Modified β2AR (Vesicle-type signals)



U-2 OS cells expressing modified beta2-adrenergic receptor challenged with various concentrations of isoproterenol.

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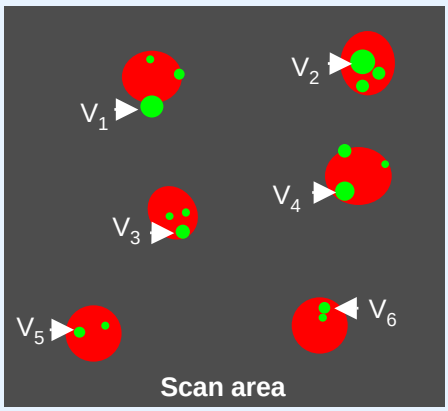
Analysis – MaxPixel Measurements

iCyte is able to track values for the brightest single pixel (the MaxPixel value). The MaxPixel-based method takes better advantage of the LSC optical scan morphometric features, resulting in a higher Z' value and a more robust assay.

We set iCyte to measure the MaxPixel value of the green fluorescence on each primary-contoured area (Figure 4A). Using the iCyte software, we excluded the MaxPixel values that reached saturation or near saturation using single-step gating (not shown). iCyte then automatically calculated the average MaxPixel value on each well. Further statistical calculation among wells and EC_{50} estimation (Figure 4B) was performed. This strategy applies only for vesicle-forming GPCRs. Note that it is possible to convert a pit-former to a vesicle-former by exchanging its C-terminal sequence in Transfluor® technology.

4A



Arrestin-GFP signal having the brightest pixel value

MaxPixel value (V_i)

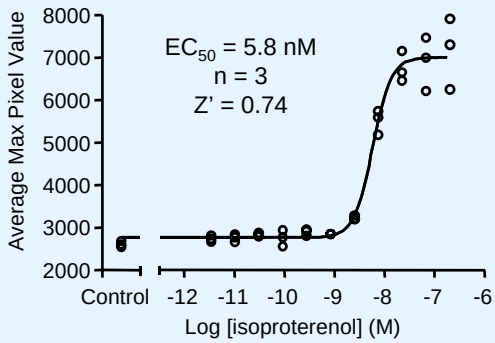
Primary contour (nuclear area)

Average MaxPixel value

$$= \frac{(V_1 + V_2 + V_3 + ... + V_N)}{N}$$

(N : number of primary contours)

4B



MaxPixel schematic for U-2 OS cells expressing modified beta2-adrenergic receptor treated with isoproterenol.

Conclusions

We designed and tested two strategies suitable for evaluating the Transfluor ® assay system with the iCyte Imaging Cytometer. These methods are simple and easy to perform, and analysis parameters can be defined by instrument operators, depending on the particular experimental situation.

On both cell lines, EC_{50} values estimated by these algorithms were in close agreement with data obtained by Norak Biosciences and previously published literature, indicating accuracy of our data obtained with iCyte technology. Thus, we conclude that the iCyte system is a suitable platform for performing a Transfluor ® assay.

References

1. Oakley RH, Hudson, CC, Cruickshank RD, Meyers DM, Payne RE Jr, Rhem SM, Loomis CR (2002) The cellular distribution of fluorescently labeled arrestins provides a robust, sensitive, and universal assay for screening G protein-coupled receptors. Assay Drug Dev Technol 1: 21-30.
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4. Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4:67-73.