

# High-Throughput Multi-parameter Single Cell Analysis

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## Introduction

The ability to perform multi-parameter assays at cellular and sub-cellular resolution is a key requirement in analyzing cellular events. Analysis at the level of individual cells is obligatory where dependent cellular processes are to be correlated, or where analyses use heterogeneous populations, e.g. primary cell cultures or transiently transfected cells. Population averaging measurements (plate readers, macro-imagers etc.) do not provide data on an individual cell basis, and hence cannot be used to correlate two or more parameters in the same cell. Conventional microscopy analysis allows resolution of multiple parameters, but does not provide the throughput required for large-scale studies. The IN Cell Analyzer 3000 (Amersham Biosciences) is a laser-based confocal line-scanning micro-imaging platform designed to address the rigorous requirements of automated live-cell fluorescence imaging. Proprietary image analysis routines compatible with the system quantify the behavior of individual cells within each image field. Here we present data from a variety of multi-parameter analyses applied to live cell assays demonstrating the benefit of sub-population and single-cell analysis in live cellular assays for drug development and functional genomics applications.

## Methods

### Sub-population analysis of multiple fluorescent reporters in heterogeneous cell populations

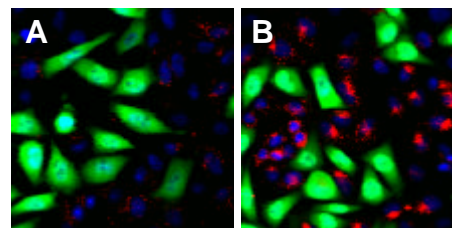
A clonal CHO-derived cell line expressing the p65 subunit of NF $\kappa$ B fused to EGFP (p65-EGFP) was mixed 1:1 with a second clonal CHO-derived cell line expressing epitope-tagged thyrotropin releasing hormone receptor (yvs-TRHR). The cell mixture was seeded to a 96-well ViewPlate (Packard). Cells were pre-loaded at 4°C with CypHer™-labelled antibody to the ysv epitope 18h after seeding, then stimulated with a final concentration of 0.05nM IL-1 $\beta$  and 40nM TRH and incubated at 37°C for 40 minutes to allow translocation of both probes to occur. Cell nuclei were stained by inclusion of 10 $\mu$ M Hoechst 33342 (Molecular Probes) in the incubation medium. Live cells were imaged using an IN Cell Analyzer 3000 fitted with a 40x objective. Images were acquired after sequential excitation—first with a 365nm laser line (Hoechst), and second with 488nm (EGFP) and 633nm (CypHer) laser lines. Each image covered an area of 750 $\mu$ m square (0.6 $\mu$ m pixels; 1.2 $\mu$ m resolution) containing an average of 590 cells. Each image was analyzed sequentially with two different image analysis algorithms (Amersham Biosciences).

### Single cell analysis of EGFP-fusion protein translocations in transiently transfected cells

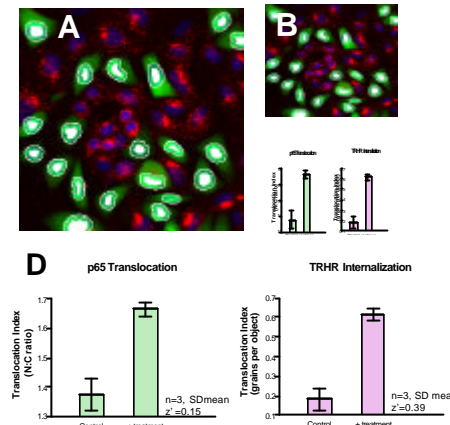
EGFP-cDNA fusions were prepared using cDNAs from GeneStorm (Invitrogen) and IMAGE collections by sub-cloning into pCORON1000 CMV driven expression vectors (Amersham Biosciences) as N- and C- terminal fusions with EGFP (Clontech). HeLa cells were transfected with cDNA-EGFP vectors in 96-well ViewPlates (Packard) using 3.5 x 10<sup>3</sup> cells per well and incubated for 24 hours before image collection. Prior to imaging, cell nuclei were stained by incubation for 10 minutes with 2.5 $\mu$ M Draq5 (Biostatus). In some cases, as indicated in figure legends, cells were also treated with 1 $\mu$ M dexamethasone for 10 minutes at 37°C prior to imaging. Cells expressing EGFP fusion proteins were analyzed using IN Cell Analyzer 3000 (Amersham Biosciences) fitted with a 40x objective. Images were acquired under simultaneous excitation with 488nm (EGFP) and 633nm (Draq5) laser lines. Fluorescence images were processed using one or more image analysis algorithms (Amersham Biosciences) to analyze automatically fluorescence intensity and distribution within each cell.

## Results

### Sub-population analysis of heterogeneous mixtures of stably transfected cell lines.

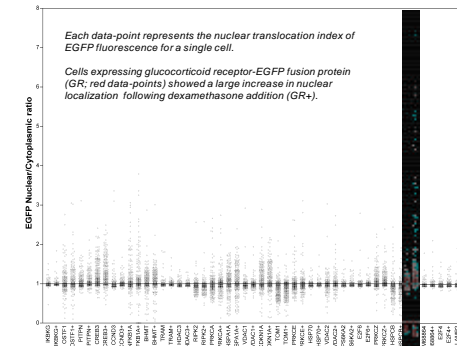


**Figure 1. Imaging of heterogeneous cell populations.** Images of mixed-cell population before (A) and after (B) treatment with a mixture of 0.05nM IL-1 $\beta$  and 40nM TRH. Hoechst dye (blue) was used to identify cell nuclei. Treatment with the agonist mixture induces translocation of p65-EGFP (green) from cytosol to nucleus. CypHer (red) fluoresces upon agonist-induced translocation to the acidic environment of the recycling endosome pathway.

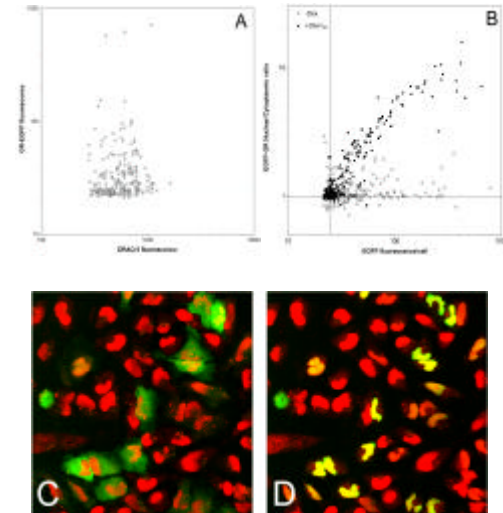


**Figure 2. Sub-population analysis.** Two distinct image analysis modules were applied sequentially to the same image data to quantify translocation of the two reporters. A. Threshold values of first module set to detect only cells expressing p65-EGFP (white bitmaps mark nuclei and cytoplasmic sampling regions); B. Threshold settings of second analysis module adjusted to detect cells expressing TRHR (white bitmap marks nucleus); C. Measurement region (inner white box) for quantification of CypHer fluorescence in selected cell from (B); D. Bar graphs of experimental results from sequential analysis.

### Single-cell analysis of reporter distributions in transiently transfected cells.



**Figure 3. Single-cell analysis.** Sub-cellular distribution of 26 distinct EGFP fusion proteins was quantified before and after (+) 10-min treatment with 1nM dexamethasone.



**Figure 4. Single-cell analysis of HeLa cells expressing glucocorticoid receptor (GR) EGFP fusion protein.** A. Expression of the fusion protein was analyzed in all cells to determine an intensity threshold (horizontal line at intensity = 25) for discrimination of expressing and non-expressing cells. B. Analysis of cellular EGFP-GR distribution before and after exposure to dexamethasone. C. Image of cells before treatment with dexamethasone. D. Image of same field of cells after dexamethasone treatment.

## CONCLUSIONS

- High speed multi-color confocal scanning supported by efficient image analysis algorithms permits high throughput multi-parameter single cell analysis in heterogeneous populations.
- Sub-population analysis allows discrimination of multiple reporters in heterogeneous cell cultures.
- Single cell analysis provides the ability to correlate reporter response with expression level enabling the use of transiently transfected cells in functional profiling screens.
- These approaches offer a very significant advance over non-imaging methods for the analysis of complex systems in drug screening and functional genomics.