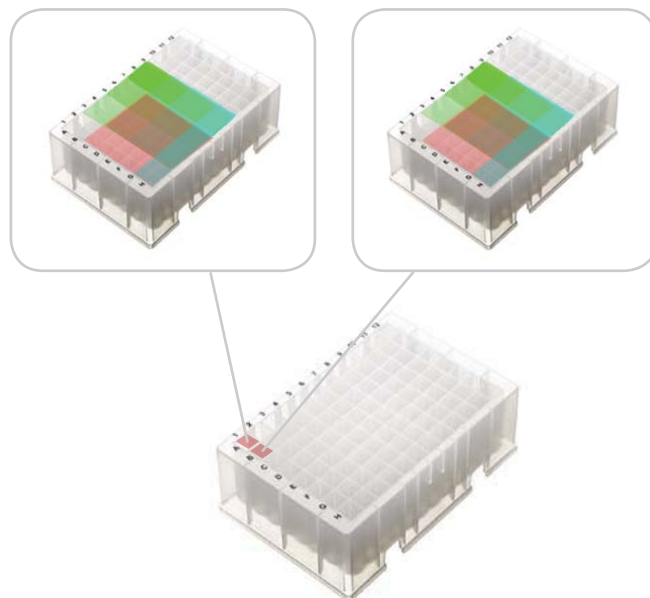


Multiplexing Signal Transduction Measurement on the ImageStream for High Content Analysis and High Throughput

IMAGESTREAM CAPABILITIES FEATURED: Internalization / Cell Signaling & Molecular Translocation / Cell-Cell interaction / Morphology / Cell Cycle & Mitosis / Co-localization / Spot Counting / DNA Damage & Repair / Cell Death & Autophagy / Immunology / Biochemistry / Oncology / Virology / Microbiology / Parasitology / Hematology / Stem Cell Biology / Oceanography / Toxicology / Drug Discovery

ABSTRACT

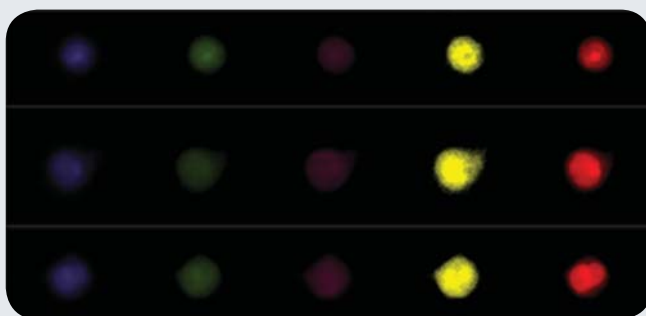
For high content analysis and screening applications including extensive dose and time course studies, increasing the number of samples which can be run simultaneously is desirable. To increase sample throughput, the fluorescent cell barcoding method previously reported by Krutzik & Nolan has been adapted for the ImageStream to be used in combination with the additional high content analysis and information gained when using imaging flow cytometry over standard flow cytometry. The method involves labeling samples with up to three different Alexa Fluor® succinimidyl ester dyes in different concentrations (i.e., intensities) followed by staining with the markers of interest. In this note nuclear translocation of NF-κB is measured in THP1 cells on a per cell basis in an automated and quantitative manner in up to 64 samples simultaneously.



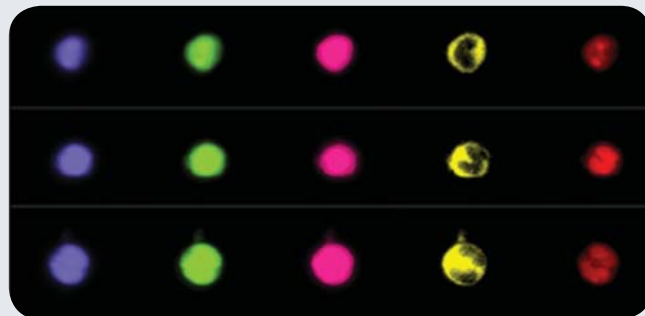
Fluorescent Cell Barcoding of each sample followed by mixing of 64 samples into one well allows the high content analysis of the ImageStream to be multiplexed for higher throughput, enabling screening applications.

Study Highlights

Representative cells from a sample imaged in multiplex exhibiting TNF-α-induced nuclear localization of NF-κB



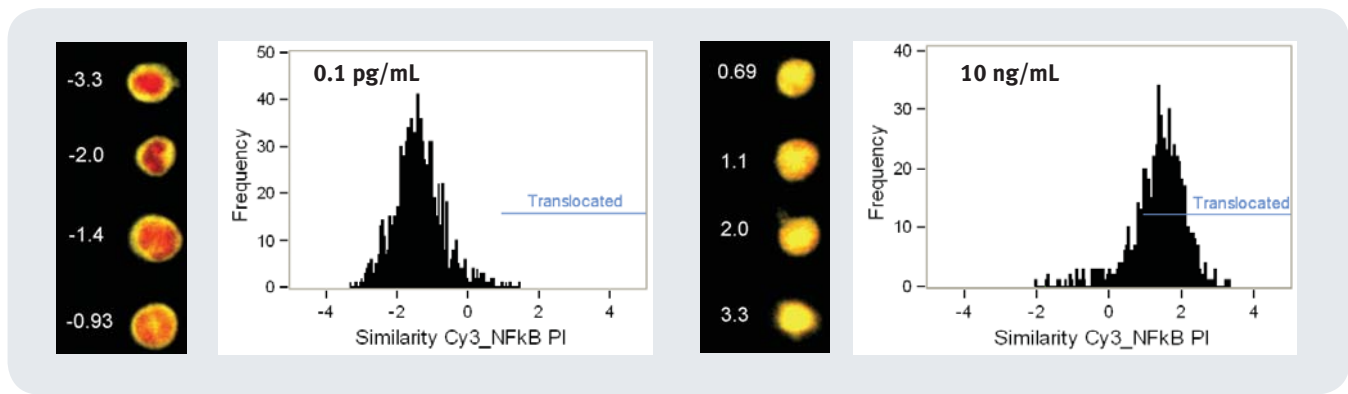
Representative cells from an untreated sample imaged in multiplex exhibiting cytoplasmic localization of NF-κB



AUTOMATED MEASUREMENT OF SIGNAL TRANSDUCTION ON A PER-CELL BASIS

In order to measure nuclear translocation of NF- κ B in response to TNF- α stimulation at various doses and exposure times, THP1 cells were stained with propidium iodide (PI) nuclear dye and a Cy3 secondary antibody to an anti-NF- κ B polyclonal antibody. Nuclear localization of the Cy3-NF- κ B was measured on a per-cell basis using the Similarity score, a measure of correlation between the NF- κ B and nuclear image pairs. The Cy3

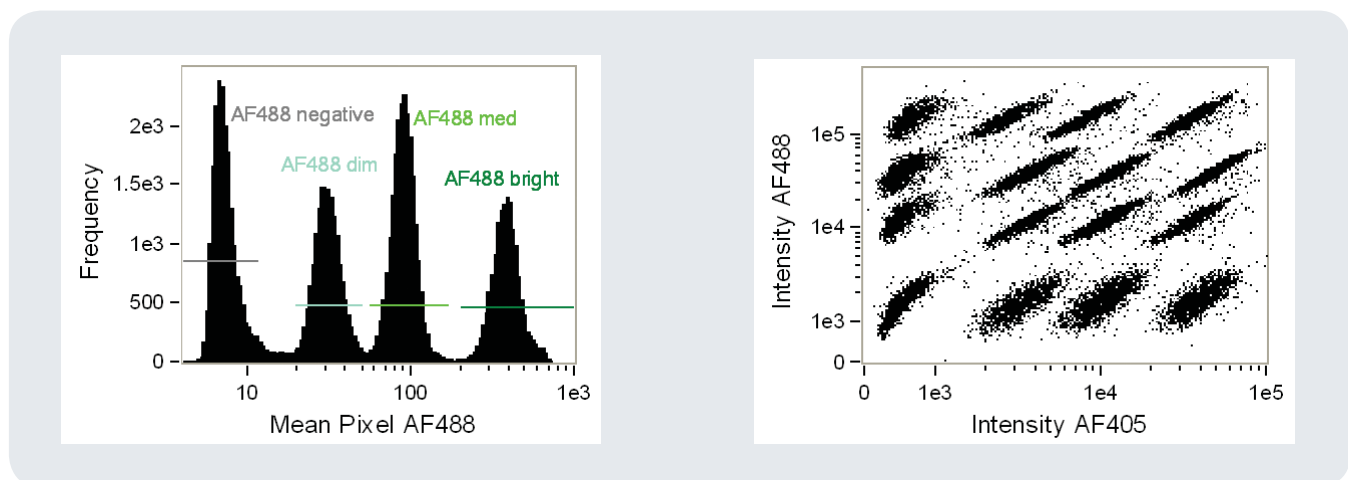
and PI images for an untranslocated cell look different from one another (left images) and thus have low Similarity values. As NF- κ B translocates to the nucleus the Cy3 and PI images look more alike, increasing the Similarity score (right images). The Similarity scores are plotted for thousands of cells from two samples, one exposed to 10 ng/mL TNF- α and the other to only 0.1 pg/mL.



FLUORESCENT CELL BARCODING FOR MULTIPLEXING 64 SAMPLES IN ONE SAMPLE TUBE

After TNF- α treatment the individual samples were stained with three different Alexa Fluor succinimidyl ester dyes (AF405, AF488, AF660), each at one of four intensities to yield 64 unique dye/intensity combinations. The samples were then mixed, stained with Cy3-anti-NF- κ B antibodies and PI nuclear dye.

The four intensities distinguished in the histogram below, extended to two dyes in the scatter plot demonstrate the ability to deconvolute 16 samples in one sample tube or well. Extending this to 3 colors at 4 intensities each allows resolution of 64 samples per well.

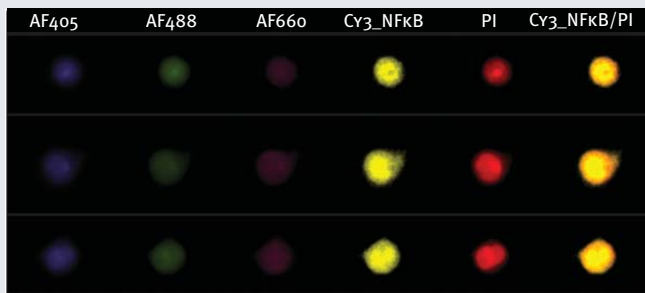


MEASURING NF-κB NUCLEAR TRANSLOCATION IN MULTIPLEXED DOSE & TIME COURSE

In this example, six doses at five time points were tested in duplicate, plus two negative control samples. Imagery of 100,000 cells (600,000 images) was acquired in approximately 10-15 minutes. The heat map table below shows the mean Similarity score (degree of translocation) for each sample after deconvolution

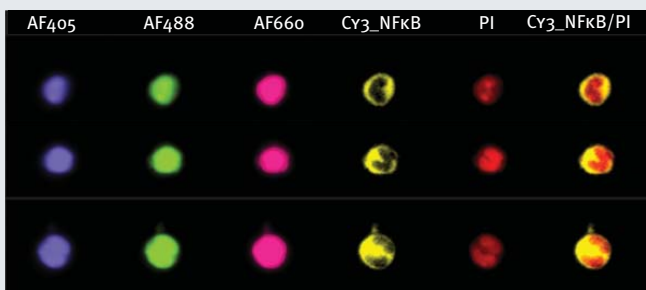
from the mixed sample. The table is organized by dose response curve (vertically) and time course (horizontally). It is apparent that doses of TNF-α above 100 pg/mL and exposure times longer than 15 minutes caused significant NF-κB translocation.

Sample 30 – 10 ng/mL TNF-α, 15 min



Dose ng/mL	60 min	45 min	30 min	15 min	5 min	0 min
10.0000	1.5236	1.5411	1.204	1.4664	0.7596	
1.0000	1.3226	1.2062	1.3096	1.2235	-0.016	
0.1000	0.9978	1.0611	0.9545	0.4996	-1.037	
0.0100	-0.3926	-0.524	-0.6951	-1.1435	-2.3559	
0.0010	-1.1153	-0.95	-1.1417	-1.2573	-1.272	
0.0001	-1.2544	-1.2401	-1.3018	-1.3137	-1.3148	-0.9818
10.0000	1.5627	1.5071	1.4382	1.3887	0.3819	
1.0000	1.1005	0.7927	1.9246	0.9966	-0.4277	
0.1000	0.945	0.688	0.6337	0.1466	-0.9696	
0.0100	-0.1469	-0.505	-0.5569	-0.9761	-1.3968	
0.0010	-1.1918	-1.1718	-1.2978	-1.3066	-1.3699	
0.0001	-0.6916	-1.3523	-0.7919	-1.4006	-1.4015	-1.3809

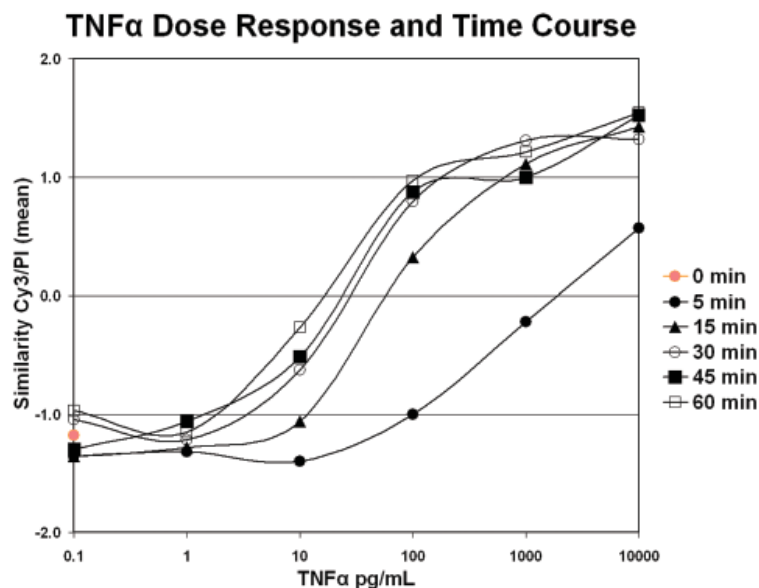
Sample 5 – 0.0001 ng/mL TNF-α, 5 min



A DOSE RESPONSE AND TIME COURSE SERIES PERFORMED IN A SINGLE TUBE OR WELL

In addition to reporting the data in heat map form, the mean Similarity score, or % Translocated for heterogeneous samples, can be plotted vs. dose as shown below for EC50 determinations. Because the many

curves shown below were obtained from a single sample well, such samples for drug screening applications could be analyzed in 96-well format to achieve high content analysis of 6,144 samples per 96-well plate.



ImageStream^X Specifications



EXCITATION SOURCES

LASER (NM)	EXAMPLE DYES
405	DAPI, Pacific Blue™
488	FITC, PE, ECD, PE-Cy5
560	Alexa Fluor® 546, Cy3
592	Texas Red®, Alexa Fluor® 594
658	Cy5, Alexa Fluor® 647, APC, APC-Cy-7

IMAGING PERFORMANCE

Magnification	20X	40X	60X
Numeric Aperture	0.5	0.75	0.9
Field of View (μ m)	120 x 512	60 x 256	40 x 170
Imaging Rate (cells/sec)	2,000	1,000	600

INSTRUMENT CAPABILITIES

Images per Cell	Up to 12
Imaging Modes	Brightfield, SSC, and fluorescent
Sample Throughput	1 sample/min nominal
Automated Processes	Startup, shutdown, and self-calibration

Pacific Blue™, Alexa Fluor®, and Texas Red® are trademarks of Life Technologies Corporation. Cy® is a trademark of GE Healthcare. ECD® is a trademark of Beckman Coulter, Inc. DRAQ5™ is a trademark of Biostatus, Ltd.