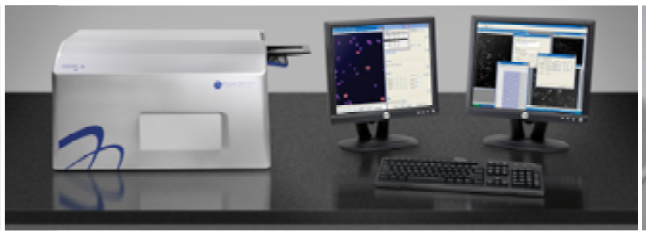

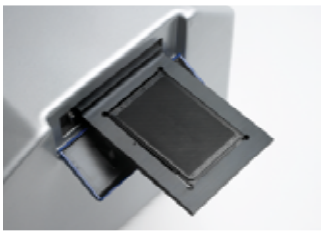

Total and Phospho-Protein Detection

p38 MAPK Activation

p38 MAPK is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases. These proteins are widely conserved among eukaryotes and are involved in many cellular processes such as cell proliferation, cell movement and cell death. It was first identified in a screen for drugs inhibiting tumor necrosis factor-mediated inflammatory responses. p38 MAPK is activated in response to a variety of stimuli including growth hormones, ligands for G-protein coupled receptors, inflammatory cytokines and stresses such as osmotic shock and heat shock. MAPKs phosphorylate specific serines and threonines on target protein substrates and function in signaling cascades that convey external stimuli from the cell surface to cellular targets such as translational machinery, cytoskeletal proteins, and transcription factors. The p38 MAPK signaling cascade is initiated when external stimuli such as growth factors, cellular stress (e.g. osmotic shock, radiation) or inflammatory cytokines are recognized by cell-surface receptors. This results in the activation of the RHO family GTPases (Rac, Rho, Cdc42), which activate MAPKKKs (e.g. MLK, TAK, ASK1). These MAPKKKs in turn phosphorylate and activate p38 MAPK kinase, which then phosphorylates p38 MAPK. Targets of phosphorylated (activated) p38 MAPK include the transcription factors STAT1, Myc/Max8, Elk-1, CHOP9, MEF2, ATF-24 and (through Msk-1) CREB. Aberrant regulation of p38 MAPK is thought to contribute to a variety of physiological pathologies, including human diseases such as asthma and autoimmunity.

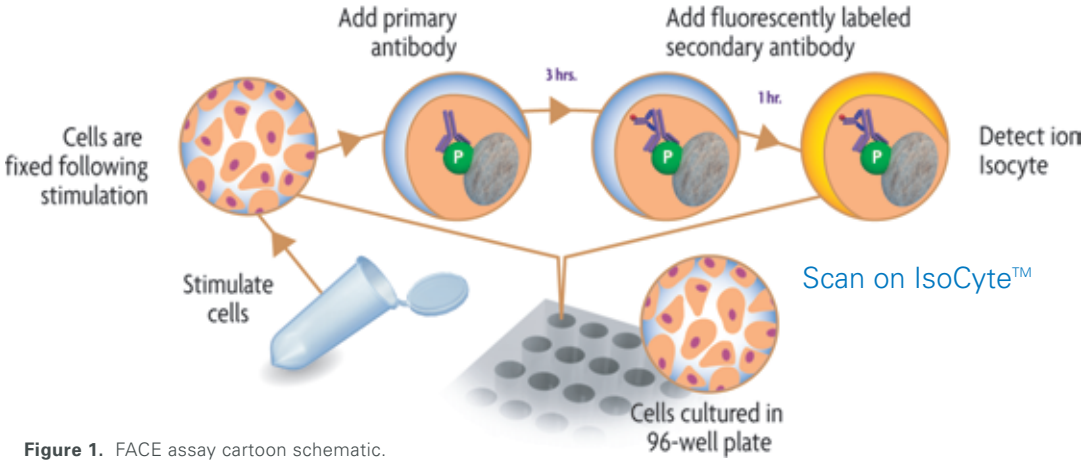

Figure 1. FACE assay cartoon schematic.

Assay Procedure

A FACE assay (Fast Activation Cell ELISA, Cat# 48600) offerred by Active Motif (Carlsbad, CA) was adapted for fluorescence read-out by replacing the HRP-conjugate with a FITC labelled secondary antibody. A cartoon depiction of the assay is shown in **Figure 1**.

Protocol

1. Seed adherent cells at ~5000 cells/well for 96-well plate, ~1500 cells/well in 384-well plate.
2. Incubate 4 hours to attach, then serum starve overnight.
3. Stimulate cells with anisomycin for 15 minutes, and fix with 4% formaldehyde for 20 minutes at RT.
4. Permeablize cells with 0.2% Triton X-100 for 10 minutes at RT.
5. Block with 5% BSA in PBS for 30 minutes.
6. Add primary antibody, seal and incubate at 4° C overnight.
7. Wash 3 times with PBS.
8. Add FITC labelled secondary antibody in 5% BSA, incubate at RT for 1hr.
9. Wash 3 times with PBS.
10. Add Ethidium homodimer-1 nuclear stain.
11. Scan on the **IsoCyte™**.

Results & Discussions

The **IsoCyte™** was configured with a 488 nm laser. The secondary antibody (green) fluorescence emission was filtered through a 510-540 nm band pass filter for channel 1 (Ch1) and the EthD-1 (red) filtered through a 600 nm long pass filter for channel 3 (Ch3). Full plate surfaces were scanned at 5 micron resolution (4 min/plate) and the resulting data was processed by BlueImage

software package. Complete wells were analyzed for individualcells. In **Figure 2** are images of cells from form wells showing the increased abundance of the phosphorylated form of p38 after stimulation as compared to the total amount of p38. These results demonstrate the use of phospho specific antibodies to detect cell signalling events mediated by kinases. A nuclear counter stain was used to count the number of cells in each well. The Ch1 signal was integrated for each well and

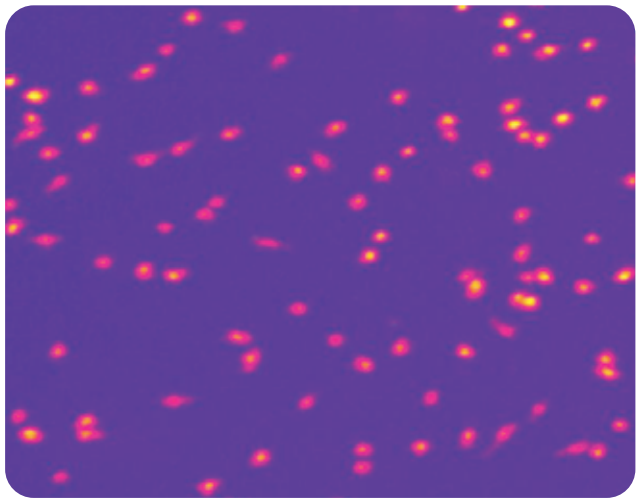

Figure 3. Cells with EthD-1 nucleic acid stain for counting

normalized to the cell count. An image of cells after staining with EthD-1 nuclear stain for cell counting is shown in **Figure 3**. Clear separation of the nuclei is found allowing robust cell counting with this technique.

A summary of these results shown in **Figure 4** demonstrate the detection of MAP kinase activation on a single cell level. The ability to measure this phosphorylation in single cells greatly improves the data quality. This assay is amenable to any protein

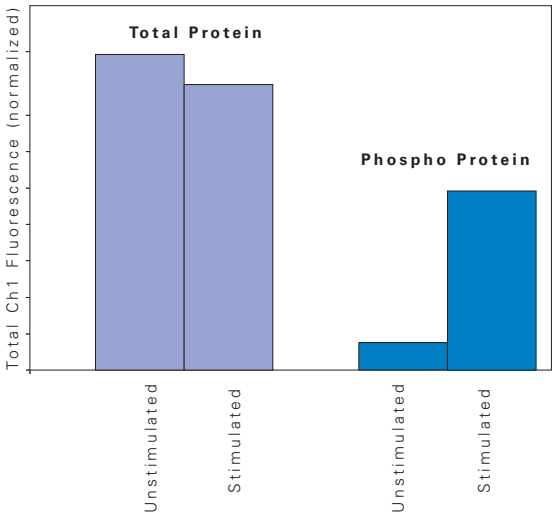

Figure 4. Plot of integrated normalized intensity for p38 assay.

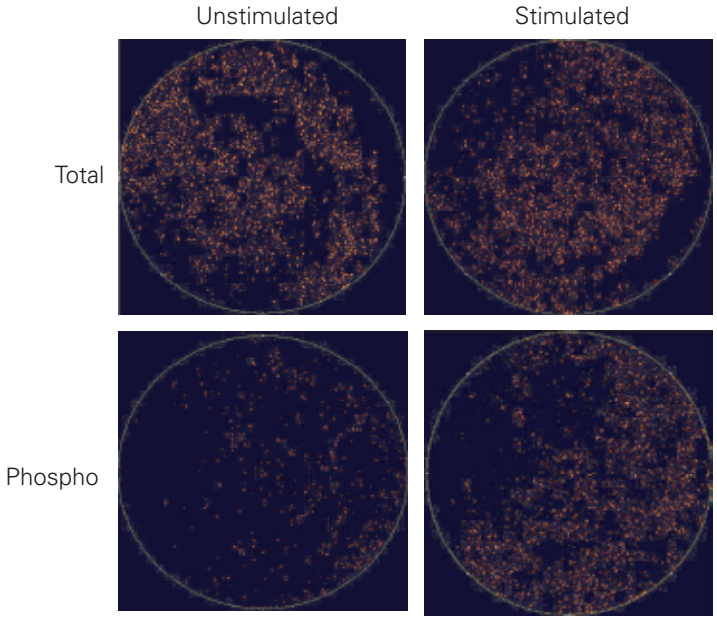

Figure 2. Raw images of cells (Ch1) displayed in BlueImage analysis software.

for which phospho specific antibodies are available. Future assays of this type will use multiple colors for higher multiplexing within the same well.

Conclusions

The use of the **IsoCyte™** scanning platform and BlueImage cell analysis software has been demonstrated for total and phospho-protein detection studies with the Active Motif Fast Activation Cell ELISA assay kit. The results show that MAP kinase activation detected on the single cell level and normalized to number of cells greatly improves the quality of data. Future assays will multiplex phospho-protein detection with total protein or other phospho species in the same well. Cell cycle analysis using propidium iodide is also feasible and can be used to look at responses within subpopulations defined by cell cycle status. In general, immunophenotyping of individual adherent cells on a whole well basis is a powerful technique in the discovery process. The **IsoCyte™** platform is an attractive solution to those laboratories in need of more user friendly quantitation of fluorescent based ELISA assays. The unique optics and scanning engine of this platform enables simple “plug and play” applications to meet the needs of both academic and biotechnology screening.

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