LanthaScreen™ Cellular STAT1/3/5 Assays For High-Throughput Screening of JAK Inhibitors

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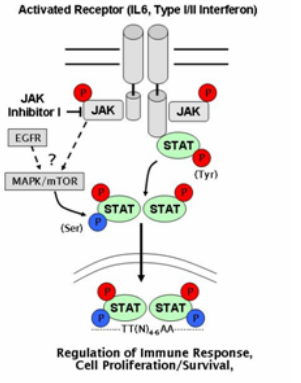
Introduction

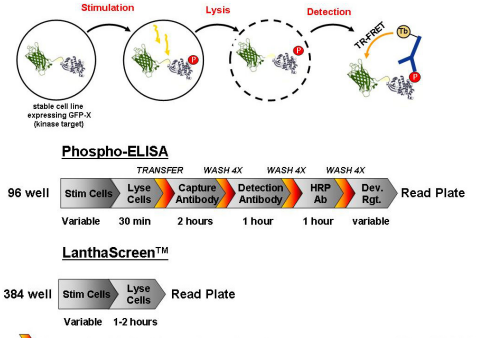
The JAK/STAT signal transduction pathway is responsible for regulation of cellular responses to a number of cytokines and growth factors. Four JAK isoforms (JAK1/2/3 and Tyk2) are responsible either alone or in combination for the activation of individual members of the STAT transcription factor family (STAT1 - 6). Stimulation with numerous proinflammatory cytokines induces the activation of JAKs ,which leads to the phosphorylation, dimerization, and translocation of STAT proteins into the nucleus. Using cell lines stably expressing Green Fluorescent Protein (GFP)-STAT genetic fusions, we have designed a number of Cellular LanthaScreen™ immunoassays which allow for the dissection of specific JAK activity in a cellular context. Specifically, we have designed HTS-compatible assays for STAT1, STAT3, and STAT5 phospho-activation. Furthermore, we have discerned the cytokine-specific role of individual JAK isoforms within a subset of these assays using Invitrogen Stealth RNAi technology. Finally, we introduce an assay capable of interrogating the activity of a constitutively-active mutant of JAK2 (V617F), which is known to be present in a large percentage of patients afflicted with myoproliferative disorders. The constitutive activity of this mutant JAK2 isoform is validated in this cell line using image-based analysis of GFP-STAT5 nuclear localization in living cells. Cellular LanthaScreen™ assays provide a robust, HTS-compatible platform for the analysis of physiological and disease-relevant JAK/STAT pathways within the cellular context in which they naturally function.

Figure 1 – JAK/STAT Signaling



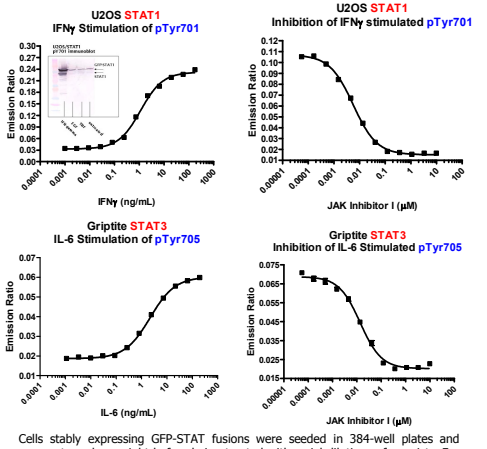
Depiction of JAK/STAT signaling. Pathway activation results in the JAK-mediated phospho-activation of STAT transcription factors. Phosphorylated STAT proteins then dimerize and translocate to the nucleus to induce target gene expression.

Figure 2 – LanthaScreen Cellular Assay Schematic



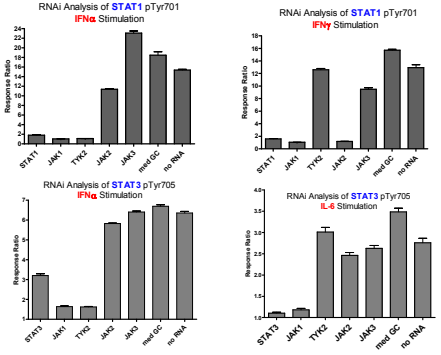
Top: Cellular LanthaScreen™ assays utilize genetic fusions of GFP with substrates known to be modified in a signal transduction pathway. GFP serves as a FRET acceptor for modification-specific Tb-labeled antibodies. Bottom: Cellular LanthaScreen™ assays offer an HTS-compatible alternative to conventional methods of phosphoprotein analysis.

Figure 3 – LanthaScreen™ Cellular Assays Measuring STAT1 and STAT3 Activation



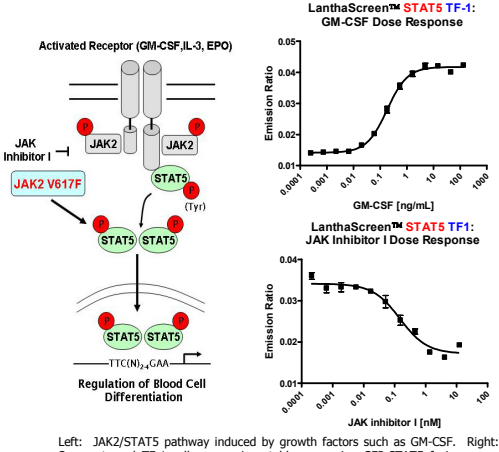
Cells stably expressing GFP-STAT fusions were seeded in 384-well plates and serum-starved overnight before being treated with serial dilutions of agonist. For antagonist experiments, cells were pretreated 1h with the indicated concentration of inhibitor before being stimulated with the EC-80 concentration of agonist. Following 30 minute stimulation with agonist, cells were lysed by addition of lysis buffer containing Terbium-labeled phospho-specific antibodies and TR-FRET signals measured on a BMG PheraStar Fluorescence Plate Reader.

Figure 4 – Discerning the JAK Isoform-Specific Role of STAT1/3 Activation using RNAi



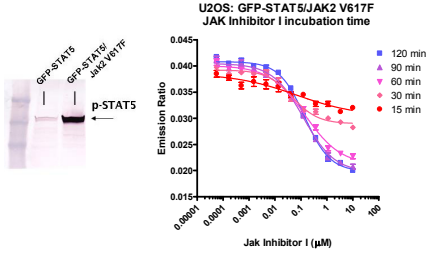
RNAi Analysis: In 96-well format, cells were transfected with the indicated Stealth RNAi oligo and incubated 60h. Following transfection, cells were stimulated with the indicated agonist for 30 minutes and samples lysed in the presence of Terbium-labeled phospho-specific antibody as indicated. TR-FRET signals were then measured on a BMG PheraStar fluorescence plate reader.

Figure 5 – LanthaScreen™ Cellular Assays Measuring STAT5 Activation in Hematopoietic Cells



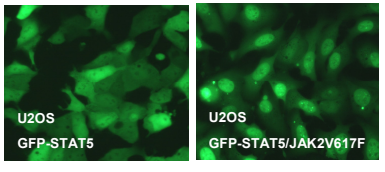
Left: JAK2/STAT5 pathway induced by growth factors such as GM-CSF. Right: Serum-starved TF-1 cells expressing stably expressing GFP-STAT5 fusions were seeded in 384-well plates and treated with serial dilutions of agonist. For antagonist experiments, cells were pretreated 1h with the indicated concentration of inhibitor before being stimulated with the EC-80 concentration of agonist. Following 30 minute stimulation with agonist, cells were lysed and processed as described previously.

Figure 6 – LanthaScreen™ Cellular Assay Using Cell Line Engineered with both GFP-STAT5 and the JAK2V617F Disease-Relevant Mutant



Left: Western Analysis of JAK2V617F-induced GFP-STAT5 phosphorylation at pTyr694. Right: U2OS cells stably expressing GFP-STAT5 and JAK2V617F were seeded in 384-well plates and serumm-starved overnight before being treated with serial dilutions of JAK Inhibitor I for the time indicated. Cells were then lysed and processed as described previously for TR-FRET measurements.

Figure 7 – Image-based Analysis of JAK2V617F-induced nuclear localization of GFP-STAT5



Left: Imaging of GFP-STAT5 in U2OS cells lacking JAK2V617F. Right: Imaging of GFP-STAT5 in U2OS cells co-expressing JAK2V617F. The presence of the constitutively-active JAK2 confers nuclear localization of STAT5, indicating consistitutive STAT5 phosphorylation.

Results and Conclusions

- **LanthaScreen™ Cellular Assays provide HTS compatible alternative for phospho-protein analysis, consistently yielding Z′ above 0.6.**
- **These assays provide a robust readout for JAK-mediated STAT1/3 phosphorylation by a variety of known pathway agonists.**
- **The JAK isoform-specific role in phosphorylation of STAT1 and STAT3 can be interrogated using RNAi with a Cellular LanthaScreen™ readout.**
- **Physiological and disease-relevant assays have been developed for the JAK2/STAT5 pathway using Cellular LanthaScreen™ technology.**

Reference: Robers et al. Anal Biochem. 2008 Jan 15;372(2):189-97